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EXPERIMENTAL VALIDATION OF NEW DRUGS FOR CALORIE RESTRICTION FROM COMPUTATIONAL MODELS

Rute Monteiro Teixeira

Dissertação

MESTRADO EM BIOQUÍMICA

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DISSERTAÇÃO ORIENTADA PELO DOUTOR JOÃO PEDRO DE MAGALHÃES E

PELO PROFESSOR DOUTOR ANTÓNIO FERREIRA

2012

Acknowledgements

I would like to thank Dr João Pedro de Magalhães for his scientific supervision, patience and understanding during this arduous process.

I would also like to thank Dr António Ferreira for his corrections, availability and encouragement.

Furthermore, I would like to thank Daniel Wuttke, for his input in the Connectivity Map analysis, as well as Dr Gillian Stepek for her invaluable help in working with *C. elegans*.

I also want to thank Inês for her unending support and friendship.

And last but not least, I want to thank my family, particularly my parents, who have always supported me in all areas in my life and helped me overcome every difficulty I have encountered in this process.

Resumo

O envelhecimento é um dos problemas mais graves que o mundo desenvolvido enfrenta no século XXI. Para a Ciência, o desafio é não só encontrar maneiras de aumentar a esperança de vida, mas também a qualidade de vida, particularmente em idades mais avançadas.

A Restrição Calórica (CR) é a limitação do consumo de calorias, sem provocar malnutrição. Há mais de 75 anos que se sabe que esta intervenção é capaz de aumentar a longevidade. Ao longo dos anos, ficou claro também que a CR diminui o risco para doenças relacionadas com o envelhecimento. Experiências num número muito variado de espécies mostraram que o efeito da CR é conservado desde a levedura até aos mamíferos. Estudos em humanos têm um grande número de limitações que não permitiram até ao momento avaliar se esta intervenção tem efeitos semelhantes em seres humanos, contudo resultados preliminares indicam que alguns factores de risco para doenças são reduzidos em pessoas sujeitas à CR.

Os mecanismos através dos quais a CR actua são ainda controversos, mas os estudos realizados até ao momento permitem discernir algumas das vias metabólicas que provavelmente mediam este processo. A evidência sugere que a via do alvo da Rapamicina (TOR), um sensor de nutrientes, está relacionada com a CR em vários modelos biológicos, tais como levedura, *C. elegans* e *Drosophila*. A inibição do TOR provoca um aumento da longevidade e visto que este acréscimo não pode ser ampliado pela CR, é provável que a via TOR seja uma das mediadoras desta intervenção. Esta via metabólica encontra-se relacionada com o desenvolvimento e o crescimento, mas é possível que em adultos, uma vez que o processo de desenvolvimento já terminou e portanto ocorre muito pouco crescimento, a activação do TOR possa desencadear o envelhecimento, fazendo sentido que a CR inactivasse este processo.

Outra via metabólica que se encontra também relacionada com a CR é a via de sinalização da insulina/IGF-1 (IIS). Estudos realizados demonstram claramente que a inibição desta via tem um efeito de aumento da longevidade, tanto em invertebrados, tais como *C. elegans* e *Drosophila*, como em ratinhos, através da activação de um factor de transcrição (FOXO) mais abaixo na cascata de sinal. Contudo, a relação entre esta via e a CR não é clara. Se por um lado a resposta à CR é diminuída quando o FOXO é inactivado, ela não é contudo eliminada completamente. Estes resultados sugerem que há apenas uma sobreposição parcial entre os dois processos.

Dado que a CR é um regime difícil de manter por seres humanos, para melhor tirar partido dos seus benefícios seria importante descobrir fármacos que possam actuar pelo mesmo mecanismo que a CR, induzindo efeitos benéficos semelhantes, chamados de miméticos da CR (CRM). Apesar de existirem já alguns fármacos que poderão ser CRMs, seria de interesse ter algum método que permitisse obter uma série de candidatos para testar.

Os objectivos deste trabalho são descobrir fármacos capazes de provocar efeitos semelhantes à CR e validar um método bioinformático para obter candidatos a CRM. Este método baseia-se numa ferramenta, o Connectivity Map (Cmap), que usa perfis de expressão génica para gerar uma lista de moléculas com actividade biológica cujo efeito no organismo correlaciona-se com um estado biológico de interesse, neste caso a resposta à CR. Após aplicação deste método, escolheram-se os fármacos mais promissores para teste dos obtidos pelo Cmap: Geldanamicina, 15d-PGJ2, Rapamicina, Radicicol, LY-294002, 17-AAG, Tricostatina A (TSA), Fulvestrant, Ácido nordihidroguaiarético e Alantoína.

Os fármacos escolhidos foram testados em *C. elegans*, um importante modelo biológico. Este animal foi utilizado em grande parte pelo seu curto tempo médio de vida, o que é bastante útil em experiências pela longevidade. Além da estirpe *wild-type* N2, recorreu-se também a uma estirpe mutante *eat-2*, com defeitos na faringe que limitam o consumo de comida, simulando um estado de CR.

Inicialmente, foi necessário optimizar as concentrações destes fármacos que permitam obter o efeito mais potente possível sem toxicidade para os animais. Verificou-se que vários destes fármacos podem ser letais em concentrações mais elevadas. Após escolha das dosagens óptimas, selecionou-se apenas quatro dos fármacos em estudo para realizar os testes de longevidade. A opção recaiu sobre a Rapamicina, o LY-294002, a TSA e a Geldanamicina.

Avaliou-se a sobrevivência das duas estirpes de *C. elegans*, simulando duas dietas diferentes *ad libitum* (AD) e CR, tanto com como sem adição destes quatro fármacos. Assim é possível não só determinar o seu efeito na longevidade destes animais como obter uma indicação da relação entre o seu mecanismo e o da CR.

A Rapamicina e o LY-294002 induziram ambas um aumento de 19.2% na sobrevivência mediana da estirpe N2, contudo não alteraram significativamente a sobrevivências dos mutantes *eat-2*. Estes resultados eram já algo esperados, uma vez que se sabia que a Rapamicina é capaz de estender a longevidade em *Drosophila* e ratinho; e o LY-294002 aumenta a sobrevivência em *C. elegans*. Mais importante é o facto de estes fármacos não

terem o mesmo efeito nos animais sob CR, possivelmente porque as vias activadas por eles já se encontram activas nestes mutantes – uma indicação de que a Rapamicina e o LY-294002 são talvez CRMs. Esta hipótese já tinha sido avançada, uma vez que o alvo molecular da Rapamicina é o TOR e o do LY-294002 é o PI-3K (parte da via IIS), ambos parte de vias que se pensa mediarem pelo menos parcialmente a resposta à CR.

Similarmente, a TSA aumenta a sobrevivência mediana dos N2 em 11.5%, mas não a da estirpe *eat-2*. Já tinha sido demonstrado que este fármaco provoca um aumento da longevidade em *Drosophila*, tornando o resultado em N2 esperado, contudo não existia até ao momento nenhum indício de que a acção deste fármaco estava relacionada com a CR. Após este resultado, pode-se propor que a TSA é possivelmente um CRM.

Já a Geldanamicina não teve qualquer efeito na sobrevivência da estirpe N2, mas diminuiu em 26.9% a sobrevivência mediana da estirpe *eat-2*, cancelando o efeito de aumento de longevidade da CR. Apesar de saber-se que a Geldanamicina tem um efeito de indução da senescência em alguns tipos de células, não tinha sido testado qual o seu efeito na longevidade. Com estes resultados, põe-se a hipótese de que a Geldanamicina, não afectando a longevidade em condições normais, impede esta se altere em resposta à CR. É possível que tal aconteça porque este fármaco actua através das mesmas vias metabólicas que a CR, mas estimulando-as a funcionarem de maneira oposta.

No entanto, em qualquer destes quatro fármacos verifica-se uma discordância com o efeito previsto para eles no Cmap. Nomeadamente, a previsão para a Geldanamicina é que tivesse um efeito semelhante ao CR, enquanto que a Rapamicina, o LY-294002 e a TSA teriam o efeito oposto. Não só isto não se verificou experimentalmente, como parece contradizer a literatura existente.

Analisou-se os genes mais significativos para as previsões do Cmap, com o objectivo de tentar compreender a razão por que as previsões obtidas são simetricamente opostas ao observado e quais as vias e metabolitos mais afectados por estes fármacos e pela CR. Tanto numa análise mais geral, com todos os fármacos com resultados mais significativos, como noutra mais específica usando apenas os quatro fármacos testados, os resultados foram semelhantes. A inconsistência em relação às previsões do Cmap não ficou completamente esclarecida, contudo esta análise apoia os resultados obtidos experimentalmente. Obteve-se ainda uma lista de genes que poderão estar envolvidos na resposta à CR, assim como no mecanismo de acção destes fármacos, o que é útil para trabalhos futuros. Muitos destes genes encontram-se

envolvidos em processos de resposta ao stress, no ciclo celular ou na regulação do crescimento e proliferação celular.

Este trabalho demonstra então que a abordagem para obtenção de candidatos a CRM usando o Cmap pode ser útil, pois os candidatos obtidos parecem estar de facto relacionados com a CR. No entanto, não é claro que a acção prevista corresponda necessariamente aos resultados experimentais. Adicionalmente, os resultados obtidos indicam que a Rapamicina, o LY-294002 e a TSA poderão ser talvez CRMs, enquanto que a Geldanamicina é capaz de cancelar os efeitos da CR, usando possivelmente as mesmas vias metabólicas.

Palavras-chave

Envelhecimento; Restrição Calórica; *Caenorhabditis elegans*; Avaliação de Fármacos; Connectivity Map; Expressão Génica

Abstract

This work assesses a new bioinformatic tool, the Connectivity Map (Cmap), in predicting Calorie Restriction Mimetic (CRM) candidates. The Cmap used gene-expression profiles to predict that the following drugs act through a mechanism strongly correlated to that of Calorie Restriction (CR): Geldanamycin, 15d-PGJ2, Rapamycin, Radicicol, LY-294002, 17-AAG, Trichostatin A (TSA), Fulvestrant, Nordihydroguaiaretic acid and Allantoin.

These drugs were tested in the nematode worm *Caenorhabditis elegans* (*C. elegans*), to optimize concentrations so as to get the strongest effect without poisoning the animals. Then the four most promising drugs were chosen for further testing: Geldanamycin, Rapamycin, LY-294002 and TSA. Lifespan assays with these drugs were performed in both wild-type (N2) and *eat-2* strains. The latter induces a CR-like state by limiting nutrient intake.

Rapamycin, LY-294002 and TSA increased median survival in N2 animals by 19.2%, 19.2% and 11.5%, respectively; but not in *eat-2*. This is an indicator that the lifespan extension induced by them may function through the same mechanisms as CR. While the Cmap predicted these drugs would have the opposite effect of CR, they were known from the literature to increase

longevity. LY-294002 and Rapamycin were even suspected of being CRM, given that their known targets are part of CR-related pathways. Therefore, these results are consistent with previous studies.

Geldanamycin had no effect on N2, but decreased survival of *eat-2* mutants by 26.9%, indicating that it might act through the same mechanisms as CR, but with an opposite effect, cancelling the CR-induced lifespan extension. This result was not expected from the Cmap results that predicted Geldanamycin to mimic CR.

Analysis of the most significant genes for the Cmap results was not able to explain the discrepancies, but corroborated the experimental data and indicated some genes, such as HSPA8 and MDM2, possibly involved in the CR and drugs' mechanisms.

Keywords

Ageing; Calorie Restriction; *Caenorhabditis elegans*; Drug Assessment; Connectivity Map; Gene Expression

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List of Abbreviations

CR – Calorie Restriction

DR – Dietary Restriction

AD – *Ad Libitum*

CRM – CR Mimetic

Cmap – Connectivity Map

C. elegans - *Caenorhabditis elegans*

TOR – Target of Rapamycin

mTOR – mammalian TOR

TORC – TOR Complex

AMPK – AMP-dependent protein kinase

IIS – Insulin/insulin-like growth factor signalling

PI-3K – Phosphoinositide 3-kinase

TSA – Trichostatin A

ROS – Reactive Oxygen Species

dilps - *Drosophila* insulin-like peptides

GH – Growth Hormone

GH-BP – GH receptor-binding protein

NGM – Nematode Growth Medium

Chapter I

Introduction

I.1 Introduction and Literature Review

Ageing is one the key issues in the twenty-first century. For the last few decades it has had a tremendous impact on human society, particularly in developed countries, (United Nations 2001) and remains a challenge for medicine and biology.

Not only is the increase of lifespan a priority, but also the increase of healthspan, as a way to achieve better quality of life in later years. As such, there is a need for biological studies with the goal of clarifying the ageing process and discovering interventions that can stop it or delay it.

This study aims to find drugs that mimic the effect of a known anti-ageing intervention, Calorie Restriction (CR), in *Caenorhabditis Elegans*, a model organism; and validate a new bioinformatic approach involving pattern-matching between gene-expression profiles for discovering new CR mimetic candidates in the future.

I.1.1 Ageing

Ageing can be defined as the changes organisms undergo with time that steadily increase the probability of dying (Lapointe & Hekimi 2010). This biological process is also called senescence. While there is ample variability in lifespan across species (de Magalhães & Costa 2009), there is also mounting evidence that ageing mechanisms are, at least partly, evolutionarily conserved (Partridge & Gems 2002; Guarente & Kenyon 2000).

Human ageing is characterized by a number of physiological changes and a general functional decline, as well as increased risk for several diseases, like cancer, diabetes, cardiovascular disease or Alzheimer's disease, for example (Gems 2011). These illnesses are usually labelled as age-related diseases. One of the hallmarks of ageing, increased inflammation levels, may play a role in several of them (Franceschi et al. 2000; Bruunsgaard et al. 2001).

While several theories have been proposed to explain the mechanism of ageing, there is still controversy regarding which one better describes this process.

Although some argue that ageing is a quasi-programmed process, i. e., an aimless continuation of the program of development growth (Blagosklonny 2006), most theories seem to focus on molecular damage over time as the main cause of ageing (F. B. Johnson et al. 1999). For the

last decades, the most accepted theory has been the free radical theory of ageing, first proposed in 1956 (Harman 1956), whose central idea is that molecular oxidative damage caused by reactive oxygen species (ROS) is the main responsible for ageing (Harman 1956; Beckman & Ames 1998). However, while it is clear that oxidative damage increases with age (Beckman & Ames 1998; R. S. Sohal & Weindruch 1996; Gustavo 2004), several studies have provided strong evidence against this theory, especially against oxidative damage being the main cause of ageing. Intervention studies designed to either increase ROS production or antioxidant protection yielded inconclusive results. Administering dietary antioxidant supplements should increase lifespan according to the free radical theory, however results in worm and fly are inconsistent and no positive evidence of this has ever been found in mammals. Moreover, some of these compounds have shown to have undesirable effects, such as increased risk of disease and even death. Overexpression of antioxidant enzymes has had similar results. The most frequently studied, SOD1, SOD2 and catalase seem to induce an increase in lifespan when overexpressed in *Drosophila*, but again this effect does not seem to be conserved in mice. In fact, even increased activity of several antioxidant enzymes simultaneously seems to have no effect on mice lifespan. Furthermore, use of SOD mimetics in *C. elegans* has also yielded mixed results, with one group reporting an increase in lifespan but four others saw no such effect, and even reported that higher doses shorten lifespan in a dose-dependent manner. However, oxidative stress resistance is effectively improved when antioxidant enzyme activity increases, which seems to indicate that ROS production is not the driving factor of the ageing process. In another approach, while knocking-out antioxidant genes has indeed produced a great number of short-lived mutants from worm to mouse, others have been reported to show no difference in lifespan. Additionally it is not clear whether the ageing process was indeed retarded or if the decrease in lifespan is the result of deleterious effects of oxidative stress. (Lapointe & Hekimi 2010; Gems & Doonan 2009).

Considering the existing evidence, there are several alternative hypothesis to the free radical theory, such as the molecular damage theory, in which oxidative damage is included but it is not the only type of damage to cause ageing (advanced glycation end-products also accumulate with ageing, accompanied by detrimental effects); or the supramolecular dysfunction theory, where the gross of the ageing process is driven by dysfunction at cell, tissue or even organ level, possibly involving insulin resistance and cell overgrowth. Finally there are still those who theorize that the ageing process might be better described as a combination of more than one of the current theories, however further studies are necessary (Gems & Doonan 2009). With the challenges arising from the decline of this theory, studies

focusing on the mechanism of known age-interventions, like calorie restriction, can help shed further light into the fundamental mechanisms of ageing.

I.1.2 *Caenorhabditis elegans*

The small free-living nematode worm *Caenorhabditis elegans* (*C. elegans*) is an important biological model. It was first proposed has a model for animal development, including neural development (Brenner 1974), due to being easy to grow and simple enough to be studied in great detail. Additionally the fact that it is transparent allows for complete mapping of each cell during development (Kimble & Hirsh 1979; Sulston & Horvitz 1977). As the years progressed however, *C. elegans* began to be widely used as a model organism in molecular biology. In genetics, specifically, it has the distinct advantage of allowing a simple and effective method of RNA-mediated interference (RNAi), for gene silencing (Fire et al. 1998; Félix 2008).

C. elegans has also been widely used in ageing studies (G. A. Walker et al. 2005), due in great part to its short lifespan: 2-3 weeks on average for the wild-type strain. This makes it easy to follow its complete lifespan (defined as the normal or average duration of life of members of a given species). The worm life cycle includes the embryonic stage, followed by four larval stages (L1-L4) and adulthood (Figure 1). While in the L2 stage, the worm can enter an alternative arrested stage, called dauer. This is a non-ageing stage that the animal can maintain when environmental conditions are unfavourable for further growth and doesn't affect post-dauer lifespan. When conditions become favourable again, the worm will develop to the L4 stage and continue regular growth (Altun & D. H. Hall 2009).

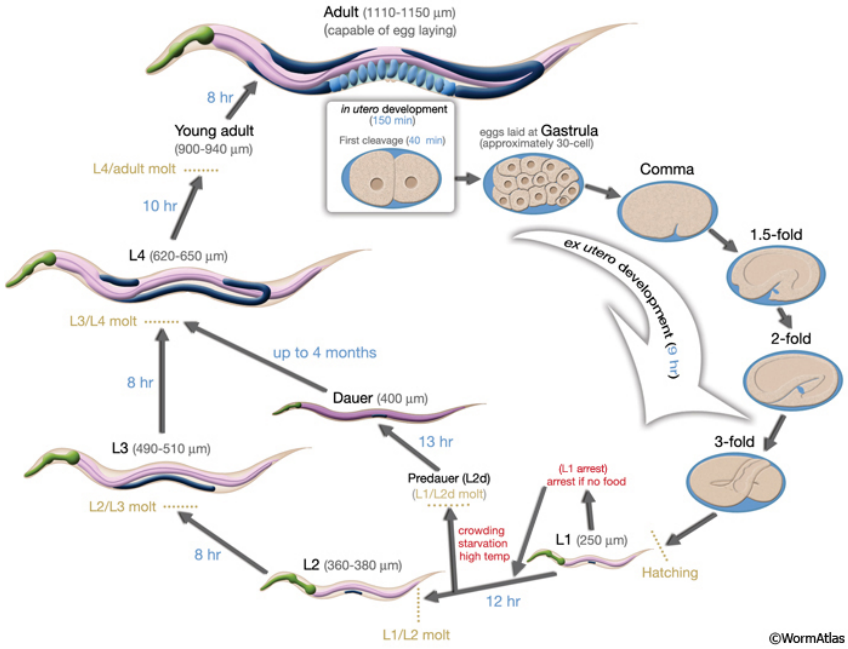

Figure 1: Life cycle of *C. elegans* at 22°C. 0 min is fertilization. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage. The length of the animal at each stage is marked next to the stage name in micrometers (µm). Adapted from Altun & D. H. Hall 2009.

In studies of calorie restriction in worms, different methods have been used. *C. elegans* feed on bacteria, so one of the most common is to simply dilute them (Klass 1977). This method has been shown to increase lifespan, so it is probable that it effectively reduces the amount of food ingested by the worm. Another common method is the use of *eat-2* mutants. These animals have altered pharyngeal function and are unable to ingest has much food as other strains. These strains also show longer lifespan than the wild-type, presumably due to their restricted diet (Avery 1993; Mckay et al. 2004; Lakowski & Hekimi 1998). In this study, *eat-2* worms were used for inducing calorie restriction.

I.1.3 Calorie Restriction

Calorie restriction (CR), also known as Dietary Restriction (DR), is the limitation of food intake below the *ad libitum* (AD) level without malnutrition. This has been consistently shown to extend the mean and maximum lifespan of a wide range of organisms, from yeast to primates, as well as delay diseases of ageing (Mair & Dillin 2008). The only exception to the rule seems to be the housefly (T. M. Cooper et al. 2004). This phenomenon was first reported in laboratory rats, over 75 years ago (McCay et al. 1935) and it remains the only environmental intervention shown to extend the longevity of both invertebrates and vertebrates (Mair & Dillin 2008). It is

widely believed that this process has evolved as a way for organisms to survive periods of food scarcity, by halting or minimizing cell division and reproduction and allocating most resources to maintenance, increasing their chances of survival. When food becomes again more widely available, reproduction can then resume in a more favourable environment (Kirkwood & Shanley 2005).

The effect of CR on lifespan has also been seen in commonly used model organisms, such as the budding yeast *Saccharomyces cerevisiae* (J. C. Jiang et al. 2000), the nematode *Caenorhabditis elegans*(Klass 1977), the fruit fly *Drosophila melanogaster* (Chippindale et al. 1993), and the rodent *Mus musculus* (McCay et al. 1935), in which it has been extensively studied (Mair & Dillin 2008; Fontana et al. 2010). This has allowed the understanding that the response of lifespan to reduced food can be graphically represented by a bell-shaped curve (Figure 2). This response has been shown in worm, flies and mice, with lifespan increasing under CR until the maximal possible value. Further starvation induces malnutrition, causing lifespan to start decreasing. In yeast, unlike what is observed in most higher eukaryotes, starvation extends chronological lifespan, no matter to what degree.

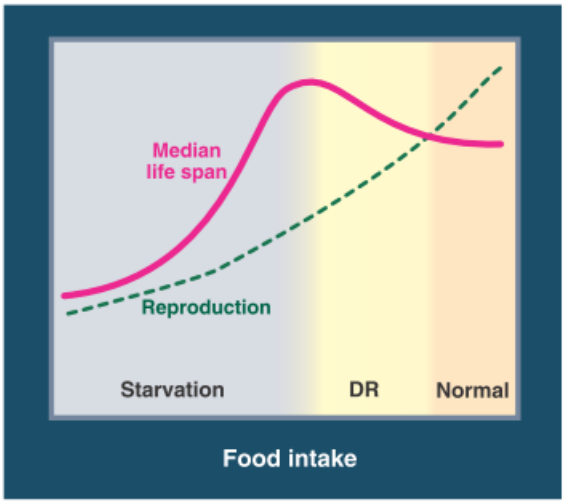

Figure 2: The effect of CR on lifespan and reproduction. Median lifespan increases as food intake is lowered from ad libitum (AD) levels until the optimization of longevity is achieved. Further restriction of food intake reduces lifespan due to starvation (***pink line***). Reproductive capacity correlates with food intake, with optimal reproduction seen at AD levels (***green line***). Adapted from Fontana et al. 2010.

While it will still be some years before we can ascertain the effect of CR in human lifespan, promising evidence has already been uncovered. In primates, specifically rhesus monkeys, CR has been shown to protect against age-related diseases and decline in function (R. M. Anderson et al. 2009; Colman et al. 2009). Human studies are undergoing, with results showing

a decrease in risk factors for age-related diseases such as diabetes, cancer and cardiovascular disease, as well as benefits against obesity, inflammation and oxidative stress among others (Fontana & Klein 2007; Fontana et al. 2004). These studies are compelling evidence of a beneficial effect of CR without malnutrition on human health, supporting the importance of further studies in this area. Understanding how CR produces these effects and how conserved these mechanisms are becomes then a fundamental step in the application of these findings.

The obvious main candidates for mediators of CR are the nutrient-sensing pathways. Studies in this area have mostly relied on testing the influence genetic mutations in the CR response. It is unlikely that a single pathway is responsible for this phenotype in any organism, as parallel and redundant pathways seem to be involved in controlling metabolism, growth and reproduction according to food intake; that is, different nutrients activate different pathways (Fontana et al. 2010). Organisms are also subjected to different degrees of starvation and their response maybe accordingly adjusted. There is evidence of this in the distinct responses to moderate and severe CR of both yeast (Bishop & Guarente 2007a) and worms (Kenyon 2010). Another aspect that has been taken into consideration is determining the level of conservation of CR mechanisms between species. Evidence shows that a number of significant pathways, further discussed below, are conserved from yeast to mammals (Fontana et al. 2010; Bishop & Guarente 2007a), with a few notable divergences. When extrapolating model organism findings for modelling human CR, it will be important to consider both different degrees of CR as well as variations between organisms.

I.1.3.1 The TOR pathway in CR

The TOR pathway, a key sensor of amino-acids and cellular energy levels, has long been connected to CR, from yeast to mice (Fontana et al. 2010). TOR is a conserved serine/threonine protein kinase, of which orthologues have been discovered in every eukaryote genome studied (Mair & Dillin 2008). This protein is fundamental in regulating growth and protein synthesis according to nutrient intake. Disruption of TOR during development leads to growth defects or, in the case of *C. elegans* larvae, even developmental arrest (Kapahi & Zid 2004; Wullschleger et al. 2006). The mammalian TOR (mTOR) binds to LST8 and to either Raptor in a Rapamycin-sensitive complex (mTORC1) or to Rictor in a Rapamycin-insensitive complex (mTORC2). mTORC1 and mTORC2 then act in distinct downstream targets, as seen in Figure 3 (Inoki & Guan 2006; Wullschleger et al. 2006).

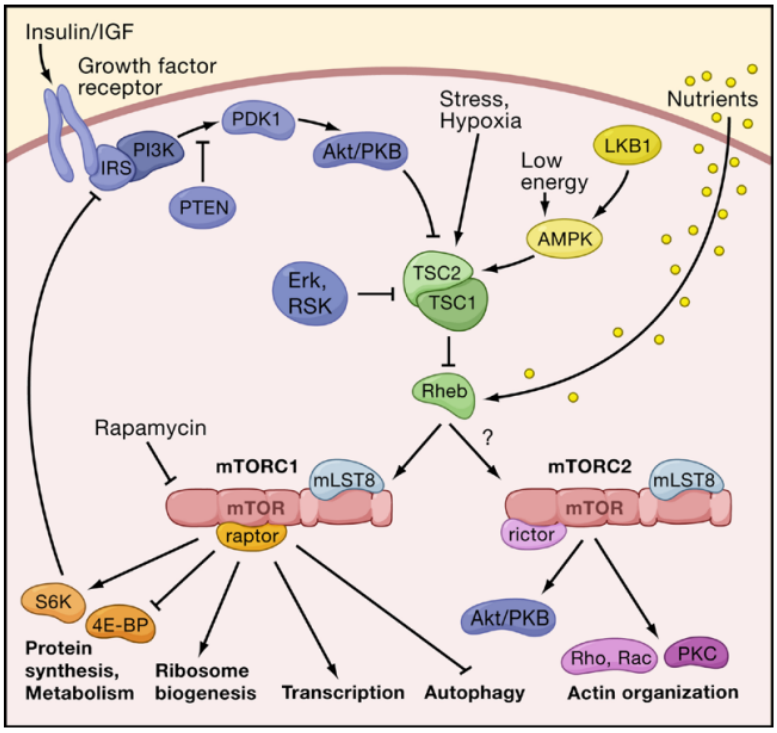

Figure 3: The mTOR signalling network in mammalians. The mTOR signalling network consists of two main branches, each mediated by a TOR-containing complex (mTORC). mTORC1 controls cell mass, while mTORC2 regulates cell shape. mTORC1 (and perhaps mTORC2) are multimeric, despite being drawn as monomers. Arrows represent activation, bars inhibition. Adapted from Wullschleger et al. 2006.

Reduced TOR signalling has been shown to extend lifespan in yeast (M. Kaeberlein et al. 2005; Powers III et al. 2006; M. Wei et al. 2009), worms (Hansen et al. 2008; Vellai et al. 2003), flies (Bjedov et al. 2010; Kapahi et al. 2004) and mice (Harrison et al. 2009; Selman et al. 2009), both by inhibition of TOR itself and of its downstream effectors. TOR affects lifespan mainly through TORC1, which is evidenced by the increase in lifespan resulting from reduced ribosomal protein S6 kinase (S6K) expression (Selman et al. 2009; M. Kaeberlein et al. 2005), one of TORC1's targets but not TORC2's. Expectedly, S6K overexpression has the opposite effect (Kapahi et al. 2004). Furthermore, mutation of *daf-15*, worm's Raptor orthologue, also increases lifespan (K. Jia et al. 2004).

Lifespan extension by reduced TOR/S6K requires alterations in protein synthesis (Steffen et al. 2008; Piper et al. 2008; Bjedov et al. 2010) and increased autophagy (Hansen et al. 2008; Bjedov et al. 2010; Amador-Noguez et al. 2007). The importance of the TOR pathway in ageing is further evidenced by the changes in lifespan as result of mutations in other proteins in this pathway, such as the AMP-dependent protein kinase (AMPK) (Mair & Dillin 2008; Bishop & Guarente 2007a), an energy-sensitive kinase that acts upstream of TOR (Figure 3)

To understand whether CR acts through the TOR pathway or in a parallel way to it, it was necessary to test if lifespan extension as result of both CR and gene expression together is the addition of what is obtained from each intervention. In case both affect the same mechanism lifespan extension from both would be similar to extension as result of each of them, while parallel mechanisms would increase lifespan independently and the outcome would be cumulative. In yeast, worms and flies with reduced TOR signalling, CR doesn't further increase lifespan (Bishop & Guarente 2007a), suggesting a shared mechanism.

Increased TOR and decreased AMPK have been implicated in tumorigenesis. Indeed some tumour cell lines are able to grow in nutrient-limited conditions by deregulating nutrient-sensing pathways, particularly by activating TOR (Mair & Dillin 2008). It is then logical to suppose that the reason CR animals have reduced tumorigenesis might be reduced TOR activity.

The connection between TOR, ageing and CR hints at a link between development and ageing. During the development phase, activation of TOR is necessary to induce growth. However in adults, where little growth occurs, a too active TOR may have deleterious effects by driving ageing. Reduction of TOR activity during adulthood is likely fundamental to increase lifespan, as done by CR.

I.1.3.1 The Insulin/Insulin-like Growth Factor Pathway in CR

The other key nutrient-sensing pathway that is a promising possibility as a CR mediator is the insulin/insulin-like growth factor signalling (IIS) pathway. It also has an important role in regulating growth and development to match resource availability (Mair & Dillin 2008). Several mutations in genes that code for components of this pathway extend lifespan robustly and in a conserved way (Figure 4 and Table 1).

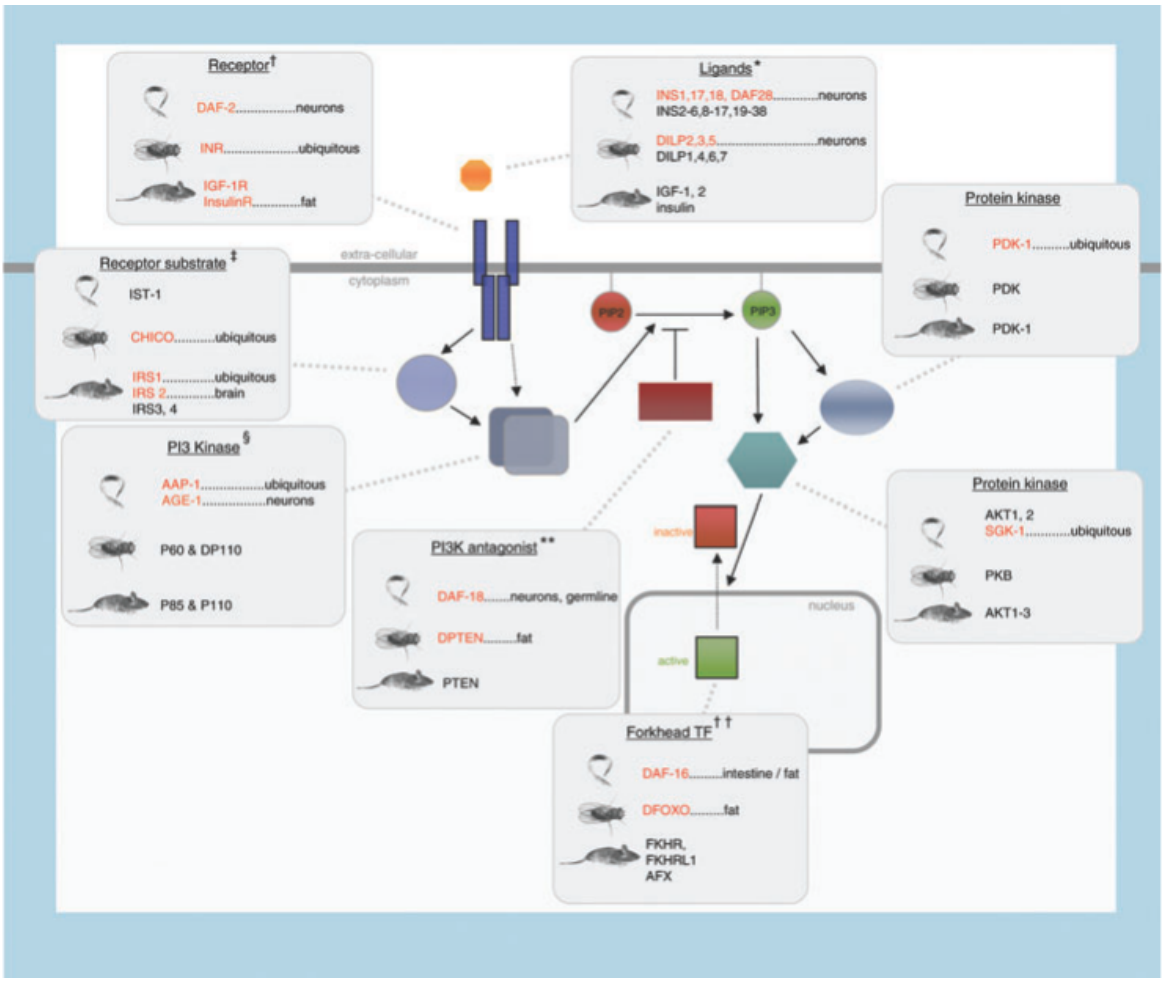

Figure 4: The insulin-like growth factor signalling (IIS) pathway components in worm, fly and mouse indicating those that extend lifespan when mutated. Species-specific names for each of the pathway components are indicated. Red text indicates long-lived mutants. More specific interventions are described in detail in Table 1. Ubiquitous means intervention affects expression of the gene throughout the whole organism. Adapted from Piper et al. 2008.

Table 1: Tissue-specific long-lived IIS mutants referred to in Figure 4. Adapted from M D W Piper et al. 2008.

Pathway component	Organism	Tissue	Intervention	Key phenotypes
***IIS ligands**	Worm	Neurons	RNAi against *ins-7*	Increased lifespan
		Neurons, vulva muscle, intestine	Overexpression of *ins-1*	Increased lifespan
		Neurons, intestine	RNAi against *ins-18*	Increased lifespan
	Fly	Neurons	Genetic ablation of the insulin producing cells	Male and female lifespan extension; reduced female fecundity; altered fat, trehalose and glycogen levels; resistant to paraquat, starvation and xenobiotics; resistant to age-related heart function decline; sensitive to heat and cold stress
†Insulin receptor	Worm	Neurons	Early adult only, RNAi	Lifespan extension; no effect on fecundity; resistant to oxidative stress
			Replacement of functional *daf-2* in neurons of mutant	Shortened lifespan (implying functional neuronal *daf-2* is required for normal signalling)
	Mouse	Fat (white adipose)	Homozygous deletion	Lifespan extension calculated using data from both sexes pooled; normal body size at birth, to smaller in late life; reduced fat mass
‡Insulin receptor substrate	Mouse	Brain	Brain-specific Irs2 heterozygotes or homozygous deletion	Male and female lifespan extension; homozygous mice infertile; increased weight and adiposity with age; insulin resistance and glucose intolerant; resisted age decline in activity
§PI 3 Kinase	Worm	Neurons	Replacement of functional *age-1* in neurons of mutant	Shortened lifespan (implying functional neuronal *age-1* is required for normal signalling)
****PI 3 Kinase antagonist (PTEN)**	Worm	Neurons & germline	*daf-18* mutation	Suppressed *daf-2* mutant longevity phenotype
	Fly	Fat	Fat-body specific induction	Male and female lifespan extension
††Forkhead transcription factor	Worm	Intestine / fat	Replacement of functional *daf-16* in intestine	Enabled IIS-mutant longevity and germline ablation longevity
	Fly	Fat	Fat-body over-expression; early adulthood only	Female lifespan increased; no effect on fecundity; Increased oxidative stress resistance

In the worm *C. elegans*, the forkhead FOXO transcription factor *daf-16* is required for the lifespan increase by reduced IIS, since the longevity extension provoked by mutations in the IGF receptor *daf-2* or in downstream signalling kinases (ex.: the PI-3K analogue, *age-1*) is completely suppressed by null mutations of *daf-16* (Kenyon et al. 1993; Weinkove et al. 2006; Ayyadevara et al. 2008; Piper et al. 2008). IIS leads the phosphorylation of *daf-16*, causing its export from the nucleus and subsequent inactivation (Mair & Dillin 2008). Inhibition of IIS, as well as stress and starvation, induce nuclear import of *daf-16*, where it regulates the expression of genes involved in a range of longevity assurance genes, most notably those involved in defensive activities such as detoxification of xenobiotics and free radicals, cellular stress response and antimicrobial activity (Fontana et al. 2010). Heat-shock factor *hsf-1* is also required for IIS-induced lifespan extension, besides affecting longevity by itself and regulating a number of other smaller heat-shock proteins (Cohen et al. 2006). Tissue-specific reduced IIS has shown that the nervous system and the gut, including the white adipose tissue of the worm, are particularly important in the connection between IIS and longevity, while *daf-16* (expressed in the neurons) coordinates the rate of ageing of the whole worm by regulation of insulin ligands (Piper et al. 2008).

As in worms, reduced IIS can also expand lifespan in *Drosophila*, as shown by mutation of the insulin receptor (Tatar et al. 2001) or its substrate, *chico* (Clancy et al. 2002). Overexpression of the fly FOXO (*dFoxo*), the *daf-16* fly orthologue, has shown that the fat body and/or gut are the tissues most implicated in this process (Fontana et al. 2010), much like what is seen in worms. As in *C. elegans*, *dFoxo* is required for most of IIS-mediated life extension in *Drosophila* (R. Yamamoto & Tatar 2011). Furthermore, deletion of *dFoxo* shortens lifespan (Min et al. 2008). The seven genes encoding *Drosophila* insulin-like peptides (*dilps*), also part of the IIS pathway, have been shown to affect longevity as well. Deletion of three of these in brain neuroendocrine cells extend lifespan (Grönke et al. 2010) and the expression of one or more is reduced in neuroendocrine cells by stress signalling and ablation of the germ line, at the same time lifespan increases (Broughton et al. 2005).

In mice, reduced activity of the IIS pathway can also increase longevity, as evidenced by the lifespan extension obtained by mutation in growth hormone (GH) and IIS genes (Bartke 2005). It should be noted that GH positively regulates IGF-1 production. These results, together with the ones for worms and flies, indicate that the link between the IIS pathway and ageing is conserved across large evolutionary distances. Increase in antioxidant defences seems to be hallmark of reduced IIS in mice. GH-deficient mice show increased expression of antioxidant enzymes and increased stress resistance in fibroblasts and muscle cells, while GH

administration decreases antioxidant defences in several organs, namely liver, kidney, muscle and heart (Brown-Borg 2007). However, there is little experimental evidence of reduction of oxidative stress having a role in lifespan extension. Mice, like other mammals, have a group of Forkhead box type O (FoxO) transcription factors homologous to DAF-16 and dFOXO, comprised by FoxO1, FoxO3, FoxO4 and FoxO6. Of these, FoxO1, FoxO3 and FoxO4 are regulated through phosphorylation by AKT/Protein kinase B (PKB) and subsequent nuclear exclusion (Kops et al. 1999; A. Brunet et al. 1999; Biggs et al. 1999), in a manner conserved from invertebrate organisms (Mair & Dillin 2008). However, there is no evidence yet of FoxO requirement for lifespan extension mediated by IIS.

A notable feature of long-lived IIS mutants is an increase in healthspan as well as lifespan. In *C. elegans*, for instance, there is evidence that IIS mutants are active until later ages (Kenyon et al. 1993; Arantes-Oliveira et al. 2003). Further evidence can be found in mice studies, like in the case of mice that lack the GH receptor-binding protein (GHR-BP), where the lifespan extension (up to 50%) is associated with a decrease in morbidity and disease-related mortality. Remarkably, in this study approximately 47% of long-lived mice died without obvious lethal pathological lesions compared to only 10% in wild-type mice (Ikeno et al. 2009). These results are similar to findings in GH-deficient mice (Bartke 2005). Both GH-deficient mice and GHR-BP knockout have increased insulin sensitivity, reduction in age-dependent cognitive impairment as well as decreased incidence and delayed occurrence of fatal neoplasms (Bartke 2005; Ikeno et al. 2009). Additionally, IRS1 (insulin receptor substrate 1) null mice display greater indices of health and lower levels of pathology at later ages, compared to controls (Selman et al. 2008). Some studies have also shown that long-lived IIS mutants in invertebrate models have protective effects against age-related pathologies, such as cancer, Alzheimer's Disease and cardiac failure (Piper et al. 2008).

Data linking the IIS pathway and CR has been conflicting. Mutations in the fly *chico* extended lifespan when nutrient intake was high, while *chico* flies under limited food conditions had lower lifespan than controls (Clancy et al. 2002). This seems to indicate that these mutant flies are already partially calorically restricted by their genotype, so when nutrient intake is lowered, they become starved. Data from worm studies, however, seem to contradict this conclusion. Since *daf-16* is required for IIS-mediated lifespan extension, CR would be blocked by removal of *daf-16* if it functioned exclusively through the IIS pathway. Yet a number of studies show that CR, even when applied using different methods, can increase the longevity of *daf-16* null mutants (Houthoofd et al. 2003; Panowski et al. 2007; T. L. Kaeberlein et al. 2006; Lakowski & Hekimi 1998). It should be noted that CR response is impaired in these cases

(Houthoofd et al. 2003; Panowski et al. 2007), suggesting a partial overlap between CR and IIS, but showing conclusively that CR is not completely dependent on IIS. Notably, *daf-16* is required for lifespan extension by sDR (a method of inducing CR by restriction of bacterial food source in solid medium) and CR applied by peptone dilution, although in these cases AMPK is required too (Greer et al. 2007; Greer & A. Brunet 2009). Lifespan extension by bacterial food deprivation requires *hsf-1* and it was shown to suppress proteotoxicity. Surprisingly, these effects were IIS independent, despite *hsf-1* being necessary for IIS-related increased longevity (Steinkraus et al. 2008). Consistent with *C. elegans* results, *dFoxo* short-lived mutant flies continue to respond to CR (Giannakou et al. 2008; Min et al. 2008). CR may also be mediated by some *dilps*, as *dilp5* has been shown to respond to CR (Min et al. 2008), indicating a connection between it and the IIS pathway. On the other hand, *dilp2* responds to dFOXO signalling but not CR (Min et al. 2008), further evidencing that the processes are not synonymous. Other strong evidence, found in both worm and fly, relates to the temporal requirements of both this processes. While CR applied late in life still induces a significant increase in longevity in nematodes (Klass 1977), flies (Mair et al. 2003) and mammals (Weindruch & Walford 1982), reduced IIS can only affect lifespan significantly when applied early in life. This has been shown in *C. elegans*, where RNAi of *daf-2* late in adulthood induces only marginal alterations in longevity (Dillin et al. 2002), and *Drosophila*, since a similar outcome was seen when *dFoxo* was expressed late in life (Giannakou et al. 2007). In rodents, there is evidence that reduced IIS may partly mediate CR. For instance, a decrease in IIS in mice confers protection against β-amyloid toxicity in a model for Alzheimer's disease (Cohen et al. 2009), in agreement with findings that CR increases insulin sensitivity and reduces β-amyloid deposition (Patel et al. 2005). Reduced incidence of tumours and spontaneous mutations in the small intestine and kidneys of CR and GH-deficient mice may also be mediated by a reduction in IGF-1 signalling (Garcia et al. 2008). Short-term starvation protects mice against chemotherapy but not cancer cells, probably by reduced IGF-1 signalling. In cancerous cells, pro-aging pathways are constitutively activity due to oncogenes, preventing the activation of stress resistance in response to CR. (Raffaghello et al. 2008; C. Lee et al. 2012). Preliminary studies in human patients indicate this effect is conserved in human beings, suggesting that short-term CR may be an effective way to prevent chemotherapy's secondary effects (Raffaghello et al. 2010).

I.1.3.3 Transcription Factors essential for CR

Transcription factors are often at the end of signalling cascades and mediate their phenotypic effects. This is also true for CR. Notably, the differential effect of CR on normal and cancer cells, mentioned in the previous section (Raffaghello et al. 2008), is mediated by the NF-E2-related transcription factor Nrf2 (C. Lee et al. 2012). Its worm orthologue SKN-1 (A. K. Walker et al. 2000) has been shown to be essential for and specific to CR-induced longevity (Bishop & Guarente 2007b). The Nrf proteins, Nrf1 and Nrf2, are involved in the response to xenobiotics and oxidative stress, by inducing expression of Phase II detoxification enzymes in the liver and digestive tract (Itoh et al. 1997; Mair & Dillin 2008). Additionally, they have a role in early development for mesendodermal differentiation (Mair & Dillin 2008). SKN-1 seems to be similarly bifunctional, being required for endodermal e mesendodermal cell fate specification early in development, while being involved in oxidative stress response during adulthood (An & Blackwell 2003; Mair & Dillin 2008). The two neurons in *C. elegans* head (ASI) and the intestinal cells are the main sites where *skn-1* is expressed. In the ASI neurons SKN-1 is expressed constitutively nuclear. On the other hand, in intestinal cells it shuttles to the nucleus under heat or oxidative stress, depending upon the p38 MAPK signalling pathway (An & Blackwell 2003; Inoue et al. 2005).

SKN-1 has been shown to be a direct target of IIS, since this pathway inhibits SKN-1 in parallel to DAF-16 (Figure 5), while a reduced IIS leads to SKN-1 nuclear accumulation in the intestine and activation of the SKN-1 target gene. Furthermore, SKN-1 extends lifespan in worm when expressed transgenically and contributes to the increased longevity and stress resistance due to decreased IIS. Remarkably, constitutively active SKN-1 delays aging independently of DAF-16 (Tullet et al. 2008), which is consistent with the observation that *skn-1* mutants, while unable to respond to CR, show increased longevity when IIS is reduced (Bishop & Guarente 2007b).

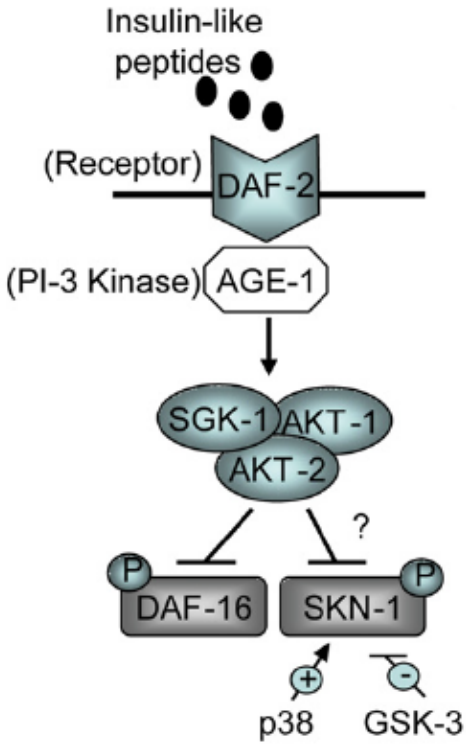

Figure 5: Model for SKN-1 inactivation by DAF-2 signalling, i.e., through the IIS pathway. Adapted from Tullet et al. 2008.

There are three different *skn-1* isoforms, *skn-1a*, *b* and *c*. The *skn-1b* isoform is expressed in the ASI neurons and it is required for CR response. When CR is induced, SKN-1b levels increase, and CR response is restored by transgenically expressed *skn-1b* in *skn-1* knockout worms, while the same is not true for other isoforms. Further proof that the ASI neurons are necessary for CR, it has been shown that laser ablation of these neurons blocks CR (Bishop & Guarente 2007b). In intestinal cells, it is the *skn-1c* isoform that shuttles between the cytoplasm and the nucleus; however this does not appear to be triggered by CR, but rather part of the response to oxidative stress (Bishop & Guarente 2007b). CR worms also exhibit an increased respiratory rate, which seems both specific of and necessary for its longevity effects. This change in mitochondrial function is dependent upon SKN-1 and the ASI neurons, specifically, while intestinal SKN-1 is not required (Bishop & Guarente 2007b).

Another transcription factor that has been shown to be essential for CR is PHA-4 (Panowski et al. 2007), the worm orthologue of the mammalian FoxA 1, 2 and 3, the FoxA family of forkhead transcription factors. Both PHA-4 and the FoxA family are essential during development, for endodermal specification of pharynx and intestinal cells in the worm (Mango et al. 1994) and for endodermal specification of the foregut in mice (Mair & Dillin 2008). However Foxa family

members seem to be bifunctional, with a second role in glucose homeostasis during adulthood (Mair & Dillin 2008). PHA-4 seems to be similarly bifunctional, since loss of *pha-4* can, by itself, suppress the extended lifespan of both *eat-2* mutants and worms subjected to CR by bacterial dilution. Its role in lifespan extension seems specific to CR, since it doesn't affect the increased longevity of *daf-2* mutants or worms with reduced mitochondrial function. Furthermore, PHA-4 role in CR is specific of its function during adulthood, as shown by the adult-specific reduction of *pha-4* expression still blocking CR (Panowski et al. 2007). It has been suggested that the DAF-16 co-regulator SMK-1 may interact with PHA-4 in mediating CR response, since SMK-1 was found to be essential for CR in a DAF-16-independent manner, as seen in Figure 6 (Mair & Dillin 2008). Given that PHA-4 and DAF-16 share DNA-binding sites (J. Gaudet & Mango 2002; Furuyama et al. 2000), it is possible that they regulate the same genes. This has been tested for the *sod* family, involved in both stress regulation and longevity. Results suggest this hypothesis is partially correct, as *sod-1* and *sod-5* are regulated by both DAF-16 in response to reduced IIS and PHA-4 in response to CR. On the other hand, *sod-3* is specifically regulated by reduced IIS/DAF-16 and both *sod-2* and *sod-4* are specifically regulated by PHA-4 in response to CR (Panowski et al. 2007).

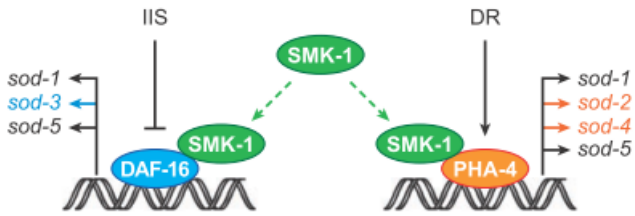

Figure 6: Forkhead box type O (FoxO) transcription factor DAF-16 and forkhead box type A (FoxA) transcription factor PHA-4 both mediate longevity pathways in the worm *C. elegans*.. SMK-1, the worm orthologue of the mammalian SMEKI (suppressor of MEK null), is essential for increased lifespan by both DR and IIS and may mediate the transcriptional activity of PHA-4, as it already has been shown to do for DAF-16, functioning as transcriptional co-regulator. Adapted from Mair & Dillin, 2008.

PHA-4 is mostly expressed in the intestine but also in a number of head and tail cells, namely neuronal (Mair & Dillin 2008). However it is not yet clear if PHA-4 response to CR is cell specific or if it acts non-cell autonomously to regulate lifespan in response to changes in nutrient intake. Furthermore, it is not known yet how PHA-4 activity is induced by CR. Nuclear/cytoplasmatic transport has been ruled out, since PHA-4 is constitutively nuclear, independent of diet (Panowski et al. 2007). Tight transcriptional regulation of *pha-4* expression may be involved, both during development and in response to CR, since transcriptional levels of *pha-4* increase twofold in both situations (J. Gaudet & Mango 2002; Mair & Dillin 2008).

Although both PHA-4 and SKN-1 have been shown to be essential for CR, independently of IIS, it is not clear which pathway(s) mediate their activity. One possible candidate is the TOR pathway, already discussed above. It would be interesting to see if changes in TOR signalling that induce lifespan extension are dependent on PHA-4/FoxA or SKN-1b/NF-E2.

While there are still significant gaps in our knowledge of pathways that mediate CR, a rough sketch of the processes involved is becoming clear and studies seem to strongly indicate that, at least some of these are evolutionarily conserved (Figure 7).

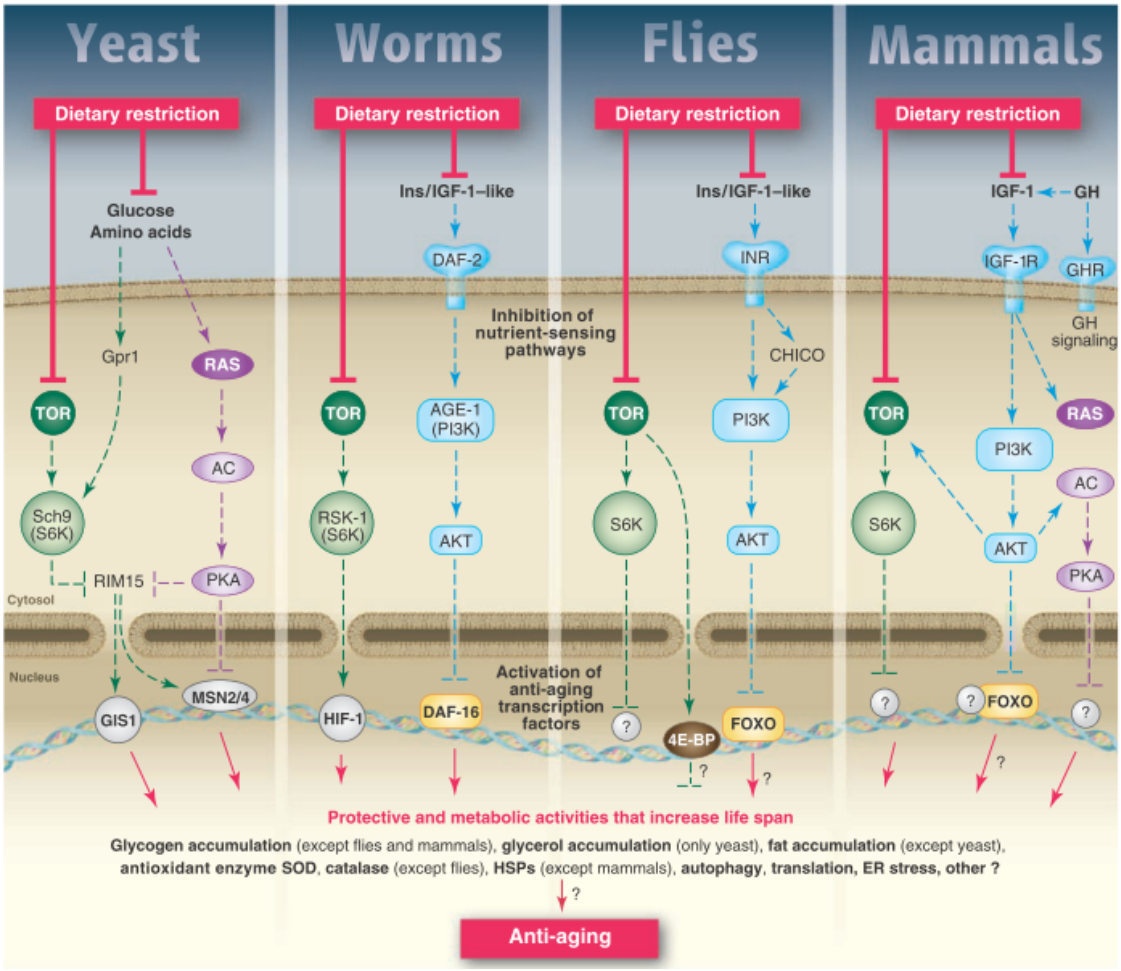

Figure 7: A model for the conserved nutrient signalling pathways that regulate longevity in various organisms and mammals. Adapted from (Fontana et al. 2010).

I.1.4 Calorie Restriction Mimetics

It is unlikely that CR could effectively delay ageing in the general population even if it is found to be effective in human beings, since people would probably not follow such a restricted diet

in the long-term (Ingram et al. 2006). The alternative is the use of CR mimetics (CRM), drugs that induce a CR-like state by affecting CR-responsive pathways. If effective, these could not only increase longevity, but perhaps more importantly, improve late-life health and well-being (Cabreiro & Gems 2010). These CRM could have one of two different effects on the CR bell-shaped curve. They can either upregulate the same pro-longevity processes as DR or limit nutrient intake or absorption (Figure 8). In the former, a greater effect on lifespan will be felt at high levels of food intake, diminishing as food intake lowers until there is no effect, as shown by the solid green line in Figure 8. This is the nutritional level where control lifespan is optimal, since longevity assurance processes are already maximized. In the latter, a greater effect will be seen at similar high food intake levels, however lifespan of CRM individuals will eventually be lower than control, since they will pushed into starvation range sooner (dashed green line in Figure 8).

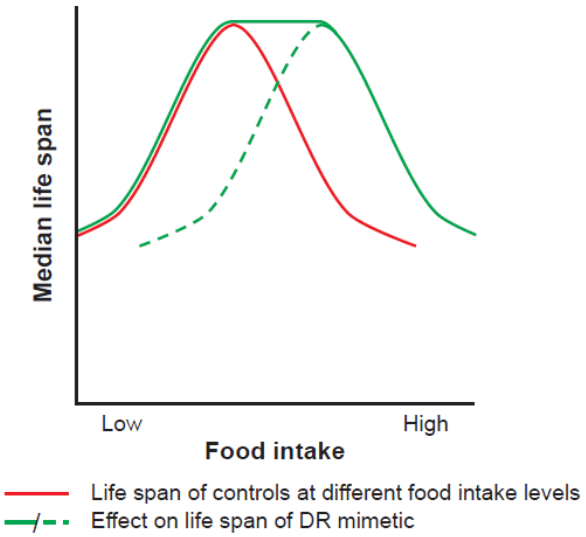

Figure 8: Effect of Calorie Restriction Mimetic (CRM) on median lifespan. Interventions that activate the same longevity assurance targets as CR will increase lifespan compared to controls at ad libitum food levels, but this effect will diminish as food intake is decreased (solid green line). Interventions that limit food uptake/cellular energy levels will increase life span at ad libitum levels but will push animals into starvation at a higher level of food intake than that which causes starvation in control animals (dashed green line). Control lifespan is shown in red. Adapted from Mair & Dillin 2008.

There is also research focussed on discovering age-suppressant drugs that do not necessarily affect CR. These drugs affect median lifespan to the same degree at all food intake levels (Figure 9), unlike CRM (Mair & Dillin 2008). An example of this is 4,4'-diaminodiphenylsulphone (DDS), that has been shown to increase lifespan in *C. elegans* and decrease ROS production, in a manner independent of both CR and IIS (S. C. Cho et al. 2010).

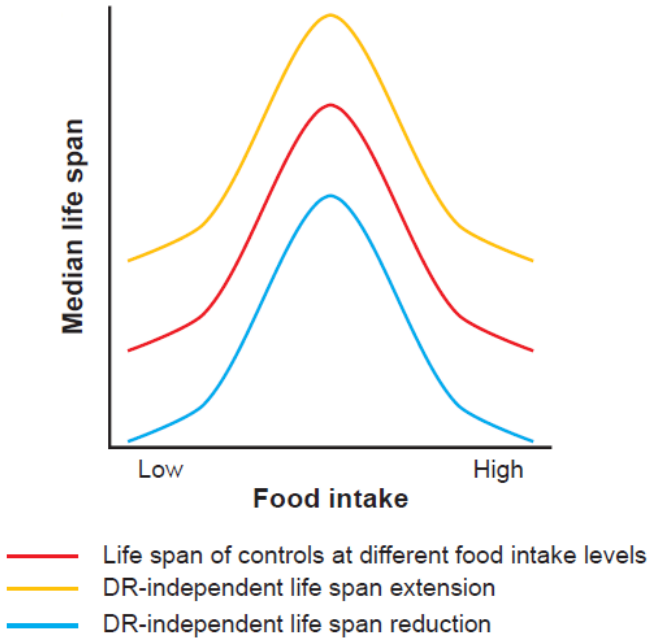

Figure 9: CR-independent age-suppressant effect on median lifespan. Interventions that affect lifespan in a CR-independent manner will either increase (yellow line) or decrease (blue line) lifespan to the same extent at all levels of food consumption. Control lifespan is shown in red. Adapted from Mair & Dillin 2008.

Current knowledge on CR pathways suggests a number of possible molecular targets for CRMs. Some drugs affecting intermediates on these pathways have already been tested, with promising results.

Metformin, a biguanide anti-diabetic drug, activates AMPK and, for this reason, was proposed as CRM. Experimental evidence has supported this hypothesis, since metformin has been shown to inhibit TOR (Kalender et al. 2010; Sahra et al. 2011), as well as induce a CR-like mRNA profile (Dhahbi et al. 2005). Notably, it can also increase lifespan and healthspan in worms, likely through a mechanism involved in CR, but independent of IIS (Onken & Driscoll 2010). Similarly, female mice fed metformin have an increase in longevity (Anisimov et al. 2008).

Another promising possibility is Rapamycin (also known as Sirolimus). This TOR inhibitor extends lifespan in flies, specifically through TORC1 and alteration to both autophagy and translation (Bjedov et al. 2010; Moskalev & Shaposhnikov 2010). Likewise, mice, even when fed Rapamycin only late in life, show a significant lifespan increase (Harrison et al. 2009). While there are data in fly that suggest this drug acts at least partly independently of CR (Bjedov et al. 2010), the fact that both share a major pathway in their mechanisms indicates that Rapamycin is still a strong candidate for a CRM and that further studies are required.

Since the Phosphoinositide 3-kinase (PI-3K) signalling cascade is also heavily involved in mediating CR, PI-3K inhibitors such as LY-294002 and wortmannin are promising CRM candidates too. Both have been shown to increase lifespan in *Drosophila* (Moskalev & Shaposhnikov 2010) and LY-294002, at least, extends lifespan in worms as well (Babar et al. 1999). While their effect on longevity is suspected to be mediated by the same pathways as CR, this is still unclear.

While there are other examples of drugs that are possible CRMs, a method for identifying CRM candidates for testing would be extremely useful. In this work, I try to demonstrate the viability of one such method.

I.1.5 Connectivity Map

The Connectivity Map (Cmap) is a bioinformatic tool, available online, comprising a collection of gene-expression profiles from human cell lines treated with bioactive small molecules and software for mining these data. It allows the discovery of connections between small molecules, drug and disease and chemical and physiological states (Lamb et al. 2006).

To use the Cmap, it is necessary to submit the gene-expression profile of cells under the condition of interest (query signature). This query contains two lists of probes (tags), one with tags whose expression is up regulated (up tags) and another with tags that are down regulated (down tags) when the biological condition of interest is induced. These tags are ordered according to the degree of differential expression against the control.

The software will then match the query to each gene-expression profile (instance) in the data set. These instances are similarly represented in a non-parametric fashion as the query, since they are each compared with the intra batch control to compile a list of genes up and down regulated by the bioactive molecule (perturbagen), and for each instance probes are rank ordered by the extent of their differential expression between the treatment and control pair. Using the Kolmogorov-Smirnov statistic, each tag list is compared to each instance, giving a measure of how comparable is the rank of a given probe in the tag list and in the instance and also how similar is the distribution of probes in both of them. Specifically, if n is the number of probe sets in the database and t is the number of probe sets in the tag list, the Cmap will construct a vector V of the position (1... n) of each probe set included the tag list in the list of all probe sets and sort these components in ascending order such that $V(j)$ is the position of

tag j, where j = 1, 2, ..., t. It will then calculate the differences between the up tags list and the instance probe set (ks^i_{up}) and between the down tags list and the same instance probe set (ks^i_{down}), using the following formulas:

$$a = \max_{1 \le j \le t} \left[\frac{j}{t} - \frac{V(j)}{n} \right]$$

$$b = \max_{1 \le j \le t} \left[\frac{V(j)}{n} - \frac{(j-1)}{t} \right]$$

... and setting $ks^i = a$, if $a > b$ or $ks^i = -b$ if $b > a$.

The comparison between the query and each instance determines whether up-regulated genes in the query match up-regulated genes in the instance and down-regulated genes in the query match down-regulated genes in the instance ("positive connectivity"), or vice-versa ("negative connectivity"). By taking into account all the results for one instance, a connectivity score ranging from -1 to +1 will be achieved. The connectivity score S^i is to zero where ks^i_{up} and ks^i_{down} have the same sign. Otherwise, S^i for the remaining instances is defined as s^i/p, when $s^i>0$, or $-(s^i/q)$, where $s^i<0$, with s^i being $ks^i_{up} - ks^i_{down}$, p the max(s^i) and q the min(s^i) across all instances. Instances are then ranked according to their connectivity scores: those most strongly correlated to the query signature are placed at the top, while those at the bottom will be strongly anti-correlated (Figure 10). With the results of every instance for one single perturbagen, the enrichment of each perturbagen is calculated, showing which induce the expression of the query signature (high positive enrichment), and which reverse the query signature (high negative enrichment). Perturbagens with zero or very low enrichment are likely to have no connection with the query signature (Lamb et al. 2006).

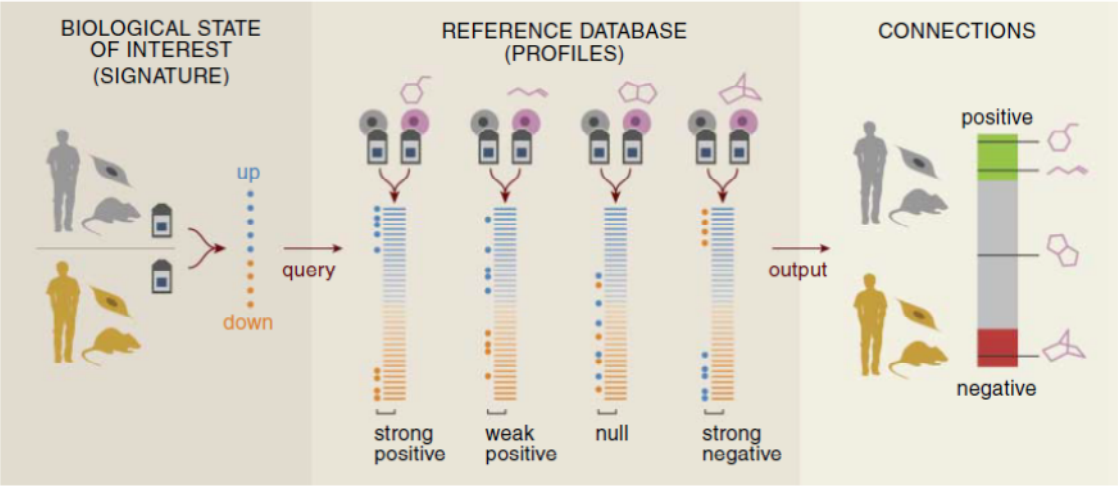

Figure 10: The Connectivity Map. Gene-expression profiles derived from the treatment of cultured human cells with a large number of perturbagens populate a reference database. Gene-expression signatures represent any induced or organic cell state of interest (left). Pattern-matching algorithms score each reference profile for the direction and strength of enrichment with the query signature (centre). Perturbagens are ranked by this ''connectivity score''; those at the top (''positive'') and bottom (''negative'') are functionally connected with the query state (right) through the transitory feature of common gene-expression changes. Adapted from Lamb et al. 2006.

Pratul Ghosh, in our group, used the Cmap to find CRM candidates (unpublished data). Using a CR signature obtained from the literature (de Cabo et al. 2003) as a query, he was able to obtain a list of drugs that are likely to act through the same pathways as CR, inducing a similar or opposite response. Analysis of the literature available for the top results shows that the following candidates (with both high positive and high negative enrichments) were the most promising for testing:

- Geldanamycin
- 15d-PGJ2
- Rapamycin
- Radicicol
- LY-294002
- 17-AAG
- Trichostatin A
- Fulvestrant
- Nordihydroguaiaretic acid
- Allantoin

The goal of this work is to test some of these predictions and establish the usefulness of this approach.

I.2 Objectives

The goals of this work are:

(1) Assessing whether candidate drugs obtained from the Connectivity Map mimic the anti-ageing effect of CR in *C. elegans*, by testing them in animals under both AD (N2 strain) and CR (*eat-2* strain) food regimens.

(2) Determining if the Connectivity Map can be a useful tool for the discovery of CRM candidates, when a CR gene-expression signature is submitted as a query.

Chapter II

Lifespan Assays

II.1 Specific Objectives

(1) Determine if the candidate drugs induce toxicity in *C. elegans*
(2) Optimize concentrations to use in lifespan assay
(3) Observe the effect of the candidate drugs in *C. elegans* lifespan, both in AD and CR conditions
(4) Determine if the drugs that affect longevity, do it through CR or other mechanism.

II.2 Material and Methods

II.2.1 Maintenance of *C. elegans* cultures

Two strains of *C. elegans* were used in this work: the wild type Bristol N2 strain, kindly provided by Dr Gillian Stepek, fed AD; and the *eat-2 (ad465)* strain, obtained from the *Caenorhabditis* Genetics Center, that is naturally under CR.

Both strains were grown using the *E. Coli* strain OP50 as food source in Nematode Growth Medium (NGM) agar plates. The OP50 is a uracil auxotroph, therefore limited in its growth. This allows for better observation and mating of the worms. The NGM (Table 2) was prepared as described by the Wormbook (Stiernagle 2006).

Table 2: Composition of NGM agar

For 1l of NGM agar	
NaCl	3g
Agar	17g
Peptone	2.5g
dH_2O	975ml
Autoclave and allow to cool	
1M $CaCl_2$	1ml
5mg/ml Cholesterol	1ml
1M Mg SO_4	1ml
1M Potassium Phosphate buffer pH 6.0 (108.3g KH_2PO_4; 35.6 g K_2HPO_4; H_2O to 1l)	25ml

The OP50 bacteria were obtained from stocks and streaked onto LB agar plates. After growing overnight at 37°C, isolated colonies are picked and inoculated in 50-100ml LB medium. After growing these cultures overnight at 200rpm, 37°C, they are stored at 4°C until required.

For maintenance of *C. elegans* cultures, they were grown in 90mm NGM plates seeded with 100µl of OP50 bacteria and kept at 20°C. The worms were transferred every 3-4 days, by chunking, to keep them from starving.

II.2.2 Bleaching of *C. elegans*

For all of the experiments regarding lifespan of the worms, it is necessary to synchronize the age of the animals tested. Commonly, cultures will have a mix of generations, making it impossible to determine a drug's effect on worm lifespan. Bleaching provides a way to obtain newly-hatched worms, all the same age.

For each strain, worms were chunked to new plates and incubated for approximately 3 days, until there were a large number of gravid adults. Bleaching should kill all larvae and adult worms, leaving the eggs (more resistant to the bleach) to originate the new synchronized culture. After washing the worms off the plates using 9ml of sterile dH_2O between two plates, the worms were transferred to 15ml Falcon tubes and centrifuged at 3000rpm, 20°C for 2min. Water was then removed to leave 3.5ml and 1.5ml of bleach (obtained by mixing domestic bleach and 5M NaOH 2: 1) was added to each tube. The tubes were vortexed for 10s immediately and again every 2min for 8min, allowing the bleach to mix with the worms and kill them. After 7min, the tubes were vortexed for another 10s and at 7min 30s they were put in the centrifuge and spun at 3000rpm, 20°C for 2min, separating the bleach from the worm eggs (pellet). The bleach was then aspirated as much as possible without disturbing the pellet and the eggs were washed twice (by adding 6ml of dH_2O, vortexing for 10s, centrifuging at 3000rpm, 20°C for 2min and aspirating the water). Finally the eggs were transferred to 50mm petri dishes containing 9ml of M9 buffer (Table 3). At this point they were left to hatch overnight in the 20°C incubator at 88rpm.

Table 3: Composition of M9 Buffer

For 1l of M9 Buffer	
KH_2PO_4	3g
Na_2HPO_4	6g
NaCl	5g
dH_2O	999ml
Autoclave and allow to cool	
1M $MgSO_4$	500µl

The next day, L1 larvae were visible swimming in the buffer. If this was not observed or there were not enough animals for the experiment, it was necessary to start over the bleaching process. If there were enough worms in the plates, they were transferred to Falcon tubes and centrifuged at 8000rpm, 20^{o}C for 5min. After separating the worms from the M9 buffer, they were pipetted onto seeded NGM plates and allowed to grow until they complete larvae development, becoming young adults, the required stage for the experiments.

II.2.3 Optimization of drugs concentration

Before proceeding to a lifespan assay, it was necessary to determine that our candidate drugs were not toxic to the model organism being used, *C. elegans*. Considering that for some drugs there was no literature available regarding its use in worms, some test concentrations were chosen, considering experiments made in other organisms using the same drug. Stock solutions for each drug were prepared by dissolving the drug in DMSO, with the exception of Allantoin that was dissolved in water.

- **Geldanamycin –** This drug is known to have been administered to *C. elegans* eggs but not to adult animals (Skantar et al. 2005). The concentrations used then were 1-100µg/ml. Since 1µg/ml of geldanamycin is almost 2µM and 100µg/ml is nearly 200µM, the test concentrations chosen for this drug were: **2µM**, **20µM** and **200µM**.
- **15d-PGJ2 –** For this drug there is no data in the literature regarding its use on *C. elegans* or even in *Drosophila*. Taking into account that 10µM was the concentration used on cell lines in the Cmap experiments, the test concentrations chosen were: **100µM**, **10µM** and **1µM**.

- **Rapamycin –** This drug was used in both cell lines at 100nM (Cmap); and *Drosophila* at 200μM (Bjedov et al. 2010) and 0.5μM (Moskalev & Shaposhnikov 2010). As such, 4 concentrations were tested in worms: **100nM**, **1μM**, **10μM** and **100μM**.
- **Radicicol –** Again, this drug was used in both cell lines at 100nM (Cmap); and *Drosophila* at 50nM-50μM (Fujikake et al. 2008). Considering this, the test concentrations chosen were: **100nM**, **1μM** and **10μM**.
- **LY-294002 –** This drug has been tested in *C. elegans* before and has shown association with longevity (Babar et al. 1999). The concentrations used ranged from 0.8μM to 2.4mM. To identify the optimal concentration, the following were tested: **10μM**, **100μM** and **1mM**.
- **17-AAG –** For this drug, the literature indicated that it had been used only in cell lines at 1μM and once at 100nM (Cmap); and in *Drosophila* at 50nM-50μM (Fujikake et al. 2008). The test concentrations chosen were **500nM**, **5μM** and **50μM**.
- **Trichostatin A –** Although this drug has been administered to *C. elegans* before, it was the L1 larvae that were exposed to it. Since the experiments in this work required it be given to adults, it was necessary to test its effects at that particular stage. The non-lethal concentrations used before were 150μg/ml (Bates et al. 2006) and 0.5-2mM (Choy et al. 2007), which led to the 2 chosen test concentrations: **100μM** and **1mM**.
- **Fulvestrant –** Fulvestrant has not been tested in *C. elegans* or *Drosophila*, so the test concentrations were chosen based on experiments in cell lines (Cmap), which were made with concentrations ranging from 10nM-1μM. Considering this, the concentrations chosen for testing were: **10nM**, **100nM** and **1μM**.
- **Nordihydroguaiaretic acid –** This drug has been used in cell lines at 1μM (Cmap); and in *Drosophila* at 1-10mM. The test concentrations chosen were then: **100μM**, **1mM** and **10mM**.
- **Allantoin –** This drug has mostly been tested topically in animals, therefore there was no studies to show its effect when ingested, which was necessary for this work. Considering it was used at 25μM in cell lines (Cmap), the test concentrations chosen were **2.5μM**, **25μM** and **250μM**.

To test these concentrations, 6-well plates were used, with 3 ml of NGM in each well. 30μl of a drug that prevents the worms from having offspring, Fluorodeoxyuridine (FUdR), was added to each well, right after the NGM. All drugs used were mixed with the medium after pouring but before it became solid, and the wells were seeded with 15μl of UV-killed OP50. To prevent bacteria from metabolizing the candidate drugs instead of the worms, they were killed using

the UV Stratalinker 2400 for 30 min, irradiating at 999900 μJ /cm^2. A sample was then taken and incubated in LB medium to make sure the bacteria couldn't grow.

Approximately 10 to 20 young adult N2 worms (2 days old) were added to each well. After 5 days of incubation at 20^{o}C, the worms were scored, i.e., checked for whether they were alive or dead. Results obtained in wells with candidate drugs were then compared to results in control wells (no drugs).

II.2.4 Lifespan Assays

In the lifespan assays, the drugs were administered to both strains of *C. elegans*. N2 were fed AD and eat-2 mutants were naturally calorie restricted, allowing for observation of the effect of the tested drugs in both regimens.

For these longer experiments, only 4 of the candidate drugs were chosen. Since Rapamycin, Trichostatin A, LY-294002 have all been linked to longevity (Bjedov et al. 2010; Moskalev & Shaposhnikov 2010; Babar et al. 1999; Harrison et al. 2009; Matuoka et al. 2003) and Geldanamycin has been shown to induce senescence (Restall & Lorimer 2010), these were thought to be good candidates for proof-of-concept of this approach. They would almost certainly induce changes in longevity in N2 worms, while their effect on *eat-2* mutants would show whether the Cmap can be useful in these type of studies.

These lifespan assays were done in 6-well plates, all with 3ml NGM and 30μl of FUdR. With the exception of control wells for both strains, each well was also added one of the candidate drugs, in the concentration chosen before. All plates were also seeded with 15μl of UV-killed OP50. 40-60 young adult worms (2 days old) of the appropriate strain were then transferred to each well. All the conditions tested were done in duplicates.

In the following weeks, worms were scored every 2-3 days for alive, dead or censored (worms that died from bursting vulva or bagging or crawled off the agar and desiccated), until there are no live animals left. 15μl of UV-killed OP50 was also added every week.

Worm survival was evaluated using the Kaplan-Meyer estimate, from which mean and median survival were calculated. Differences between survival curves were assessed using a Log-rank test.

II.3 Results and Discussion

II.3.1 Optimization of drugs concentration

The worms were observed moments after transfer and 5 days later. In the control well, all worms were alive. In the wells with drugs, it should be noted that in some cases (particularly for high concentrations) worms were dead moments after the transfer. The full results are shown in Table 4.

Table 4: Results for optimization of drugs concentrations, tested in N2 *C. elegans*

Drug	Concentration (μM)	Observations
Geldanamycin	200	All dead after transfer
	20	No dead
	2	No dead
Rapamycin	100	All dead after transfer
	10	No dead
	1	No dead
	0.1	No dead
Trichostatin A	1000	All dead after transfer
	100	No dead
LY-294002	1000	All dead after transfer
	100	No dead
	10	No dead
15d-PGJ2	100	Most dead after 5 days
	10	No dead
	1	No dead
Radicicol	10	All dead after transfer
	1	No dead
	0.1	No dead
17-AAG	50	All dead after transfer
	5	No dead
	0.5	No dead
Fulvestrant	1	No dead
	0.1	No dead
	0.01	No dead
Nordihydroguaiaretic acid	10000	All dead after transfer
	1000	No dead
	100	No dead
Allantoin	250	No dead
	25	No dead
	2.5	No dead

The optimal concentrations (Table 5) were the highest ones for each drug that were not toxic for the worms, i.e., that did not induce death. The goal is to induce the strongest effect in longevity possible, without inducing toxicity.

Table 5: Optimal concentrations for each candidate drug

Drug	Optimal Concentration (μM)
Geldanamycin	20
Rapamycin	10
Trichostatin A	100
LY-294002	100
15d-PGJ2	10
Radicicol	1
17-AAG	5
Fulvestrant	1
Nordihydroguaiaretic acid	1000
Allantoin	250

While this experiment does not allow determination of how much of the drug accumulated in the worm tissue or if it was absorbed at all, the fact that some drugs at high concentrations induced toxicity is proof of at least partial absorption of the drug. This is particularly important since worms are remarkably resistant to pharmacological perturbation (Burns et al. 2010), due to the physical characteristics of its digestive system (Cox et al. 1981; Avery 2003) and its powerful xenobiotic enzymes (Lindblom & Dodd 2006).

II.3.2 Lifespan Assays

Rapamycin (Figure 11), LY-294002 (Figure 13) and Trichostatin A (Figure 12) increased lifespan in N2 worms. Geldanamycin, on the other hand, has no effect on longevity of N2 worms and seems to abolish the CR-induced lifespan extension (Figure 14).

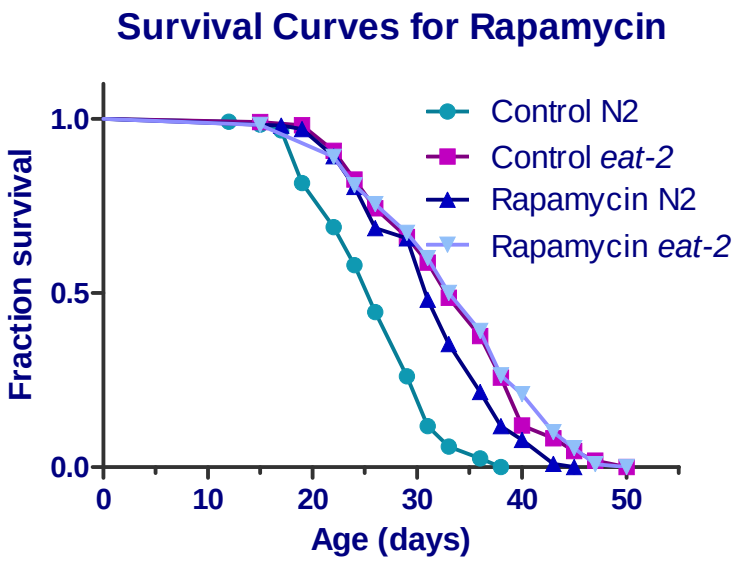

Figure 11: Kaplan-Meier curve for Rapamycin. N2 and *eat-2* worms were either treated with Rapamycin or not treated at all. Duplicates with similar results were pooled. For additional data, see Table 6.

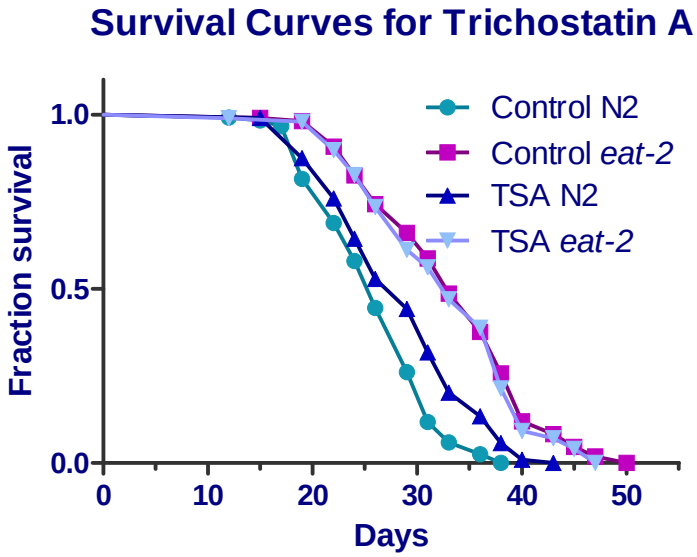

Figure 12: Kaplan-Meier curve for Trichostatin A. N2 and *eat-2* worms were either treated with Trichostatin A or not treated at all. Duplicates with similar results were pooled. For additional data, see Table 6.

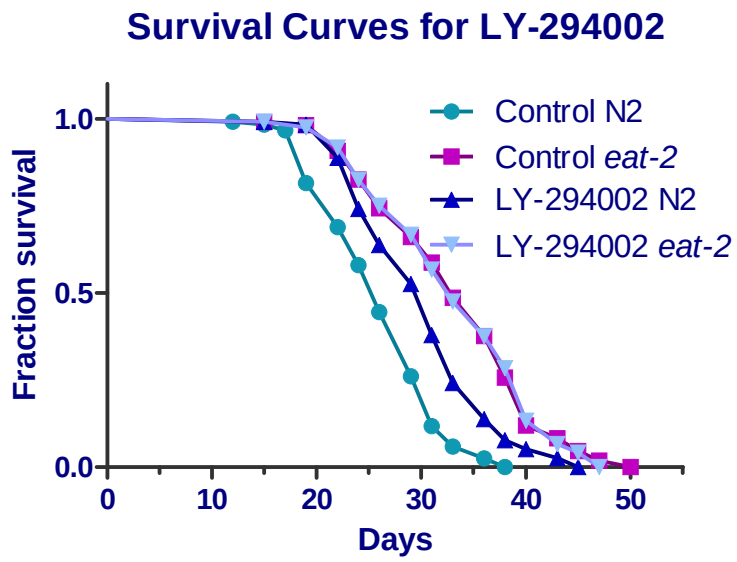

Figure 13: Kaplan-Meier curve for LY-294002. N2 and *eat-2* worms were either treated with LY-294002 or not treated at all. Duplicates with similar results were pooled. For additional data, see Table 6.

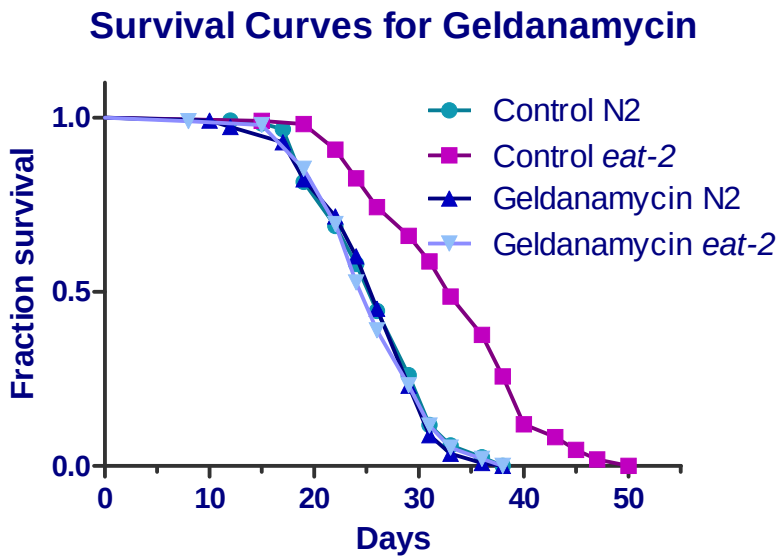

Figure 14: Kaplan-Meier curve for Geldanamycin. N2 and *eat-2* worms were either treated with Geldanamycin or not treated at all. Duplicates with similar results were pooled. For additional data, see Table 6.

The results for the effect of Rapamycin, LY-294002 and Trichostatin A in N2 (fed AD) are consistent with previous work. Both Rapamycin and Trichostatin A have been shown to increase lifespan in other organisms (Bjedov et al. 2010; Moskalev & Shaposhnikov 2010;

Harrison et al. 2009; Matuoka et al. 2003). Moreover LY-294002 has been linked to longevity in *Drosophila* (Moskalev & Shaposhnikov 2010) and in *C. elegans* (Babar et al. 1999). However that same increase does not happen with the *eat-2* mutants (under CR), since lifespan of *eat-2* treated with these drugs is not significantly different from the lifespan of the *eat-2* control (Table 6).

Table 6: Effect of Rapamycin, Trichostatin A, LY-294002 and Geldanamycin in *C. elegans* N2 (fed AD) and *eat-2* mutants (in CR)

Strain/Treatment	Median Survival (days)	Mean Survival (days)	P-value vs. Control N2[a]	P-value vs. Control *eat-2*[a]	Number of animals[b]
Control N2	26	25			119
Control *eat-2*	33	32.3			109
Rapamycin N2	31	30.3	< 0.0001		102
Rapamycin *eat-2*	34.5	32.4		n.s.	110
Trichostatin A N2	29	27.2	0.0004		104
Trichostatin A *eat-2*	33	31.8		n.s.	98
Geldanamycin N2	26	24.7	n.s.		113
Geldanamycin *eat-2*	26	24.5		< 0.0001	95
LY-294002 N2	31	29	< 0.0001		116
LY-294002 *eat-2*	33	32.2		n.s.	120

[a]P-values were calculated using a Log-rank test. P-values below 0.05 are considered significant, indicating that the two lifespan are different. n.s. stands for not significant.
[b]Number of animals is the total of the population as well as the number of deaths observed, since there were no censored animals in this experiment.

Out of the 3 drugs that increase lifespan in N2 animals, Rapamycin and LY-294002 show the greatest effect, both with a 19.2% of increase in median survival, while TSA induced only a 11.5% increase.

Since lifespan under CR is not affected by these 3 drugs, there is indication that these 3 drugs might increase lifespan through CR. This is somewhat expected, particularly with Rapamycin, which has been shown before to mimic CR's anticancer effect (Lashinger et al. 2011; Nogueira et al. 2012). Both this drug and LY-294004's known targets, TOR and PI-3K respectively, are part of pathways long known to be involved in CR (Mair & Dillin 2008), which made them strong candidates for CR mimetics even before this study (Blagosklonny 2010; Blagosklonny

2009; Cabreiro & Gems 2010). As such, these results show the usefulness of the approach used in this work.

More striking perhaps are the results obtained for Geldanamycin. Considering that in some situations it has been known to induce senescence (Restall & Lorimer 2010), it could be expected to decrease lifespan in general. However, N2 lifespan remains the same after treatment with this drug. On the other hand, *eat-2* lifespan is greatly reduced, with a 26.9% decrease in median survival, cancelling out the lifespan-extension effect of CR. This seems to indicate that Geldanamycin affect the same pathways as CR, but with an opposite result.

It should be noted, however that these results seemingly contradict the Cmap output that is the basis of this experiment. Although these drugs were strong candidates for affecting the same pathways as CR, the proposed effect is symmetrical to the observed for all of them. This may be related to the fact that the Cmap gene expression profiles are from human cell lines, while these experiments were done in *C. elegans*. Further analysis of the CR database of experiments and the results obtained from it is necessary.

Chapter III

Connectivity Map Analysis

III.1 Specific Objectives

(1) Determine which genes of the CR signature input into the Connectivity Map have the most impact on the results
(2) Understand why the Connectivity Map results seem to indicate symmetrical effects to what the literature and my own experiments show

III.2 Materials and Methods

III.2.1 Analysis of significant genes in the top results vs. CR signature

Since the experimental data above and the Cmap predictions are not consistent, analysis of the genes in common between the database and the query signature can perhaps shed light on this inconsistency. These genes should justify the Cmap results and provide clues to which pathways are involved.

The Connectivity Map allows the download of the database of gene expression profiles for different drugs it uses to compare to a query signature. From this database, the data for my top candidates were taken.

For each drug, the Cmap has several gene expression data sets, called instances. To analyze each instance on its own would take too long and the results might highlight the bias or errors of a specific instance, which is diluted when they are analyzed together. For these reasons, the data for each drug was reduced to one gene expression value per probe, by calculating the median of the values for each probe. The probes symbols were then substituted by their respective gene symbols, so the end results of the analysis would be easier to interpret.

These drugs were divided into 2 groups: the ones with a positive enrichment (indicating they have a similar effect in gene expression as CR) and the ones with a negative enrichment (meaning that they have the opposite effect as CR). Furthermore, the top (overexpressed) and bottom (down-regulated) 10 genes for each drug were taken. These genes show the bigger change in expression from the treatment with the drugs, therefore have probably more influence in the results obtained from the Cmap, making them the most suitable for this analysis.

The chosen genes were then compared to the CR signature used to obtain these candidates, specifically to check if their expression is changed in the same way by the drugs and by CR or

not (that is, if they are up or down regulated in both cases or if one is up regulated and the other down regulated). Additionally, seeing as several of these genes appeared in the top and/or bottom 10 of a number of these drugs, the number of times they appeared was counted and used as measure of their significance for the overlay between drugs and CR signature. They were then ranked accordingly, from the most often found to least. This analysis was done using a Python script.

III.2.2 Analysis of significant genes for tested drugs vs. CR signature

To determine the most significant genes in the results obtained for the tested drugs and from there infer which pathways might mediate their effects, a specific analysis was performed. For each drug the 5 genes most significantly overexpressed and the 5 genes most down-regulated were chosen, since they are more likely to have a greater weight in the Cmap result. As in the previous analysis, the chosen genes were compared to the genes in the CR signature, using a Python script.

III.3 Results and Discussion

III.3.1 Analysis of significant genes in the top results vs. CR signature

The predicted results for drugs with positive enrichment were that the top-ranked genes' expression would vary in the same way for the drugs and for CR, either being up-regulated in both cases or down-regulated for both also. On the other hand, for the negative enrichment drugs, the reverse was expected, with the expression top-ranking genes changing under CR in the opposite way to what happens with the drugs (one is down-regulated while the other is up-regulated and vice-versa). These genes would then be the main common link between these drugs and the CR gene expression profiles, providing a starting point for understanding which pathways are at work in both cases.

However, the results were quite different from what was predicted. For positive enrichment drugs, like Geldanamycin, 9 out of the top 10 results show opposite behaviour between drugs and CR (Table 7).

Table 7: Top 10 ranking genes from the comparison of positive enrichment drugs with the CR signature

Gene	N[a]	CR vs. Drugs[b]
MDM2	12	CR↑ Drugs ↓
CAPRIN1	10	CR ↓ Drugs ↑
DNAJB6	9	CR ↑ Drugs ↓
TFPI	9	CR ↓ Drugs ↑
DNAJB6 /// TMEM135[c]	8	CR ↑ Drugs ↓
ING1	8	CR ↑ Drugs ↓
RBBP4	8	CR ↓ Drugs ↑
HSPA8	7	CR ↑ Drugs ↓
SETDB1	7	CR ↑ Drugs ↑
UBB	7	CR ↑ Drugs ↓

[a]N stands for the number of drugs with positive enrichment (out of a total of 14) in which the gene was either in the top or bottom 10.

[b]Compares behaviour of genes under CR with the effect of the drugs with positive enrichment. Arrow direction shows whether the gene is up or down-regulated in each situation.

[c] Different genes that bind to the same probe.

The complete results for drugs with positive enrichment can be found in Appendix 1.

Furthermore, the drugs with negative enrichment also yielded unexpected results, with 9 genes having the same behaviour under the effect of the drugs and under CR in the top 10 (Table 8).

Table 8: Top 10 ranking genes from the comparison of negative enrichment drugs with the CR signature

Gene	N[a]	Drugs vs. CR[b]
ARNT	9	CR ↑ Drugs ↑
TFPI	9	CR ↓ Drugs ↓
MDM2	8	CR ↑ Drugs ↓
CXXC1	8	CR ↑ Drugs ↑
MOGS	8	CR ↑ Drugs ↑
ACTG1	8	CR ↓ Drugs ↓
SETDB1	7	CR ↑ Drugs ↑
ING1	7	CR ↑ Drugs ↑
DNAJB6 /// TMEM135[c]	6	CR ↑ Drugs ↑
KPNB1	6	CR ↑ Drugs ↑

[a]N stands for the number of drugs with negative enrichment (out of a total of 12) in which the gene was either in the top or bottom 10.
[b]Compares behaviour of genes under CR with the effect of the drugs with negative enrichment. Arrow direction shows whether the gene is up or down-regulated in each situation.
[c]Different genes that bind to the same probe.
The complete results for drugs with negative enrichment can be found in Appendix 1.

While this analysis does not clarify the discrepancy observed between the Cmap data and the experimental results, it shows that experimental data from this study is coherent with the microarray data in the Cmap database. It is possible that an analysis using the full set of genes, like the Cmap logarithm performs, will yield a different result, therefore explaining inconsistencies still remaining.

The functions of the genes obtained in this analysis (Table 9) can be clues regarding what pathways are being activated or repressed by both these drugs and CR, as well as possible candidates for future experiments.

Table 9: Function of the genes obtained in Table 7 and Table 8.

Gene Symbol	Gene Name	Function
MDM2	Mdm2 p53 binding protein homolog (mouse)	Target gene of transcription factor tumour protein p53 (TP53). Encodes a nuclear phosphoprotein that binds and inhibits transactivation by TP53, as part of an autoregulatory negative feedback loop
CAPRIN1	cell cycle associated protein 1	Not known

DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	Molecular chaperone involved in a wide range of cellular events, such as protein folding and oligomeric protein complex assembly
TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	Protease inhibitor that regulates the tissue factor (TF)-dependent pathway of blood coagulation
TMEM135	transmembrane protein 135	Not known
ING1	inhibitor of growth family, member 1	Tumour suppressor protein that can induce cell growth arrest and apoptosis, physically interacts with TP53 and is a component of the p53 signalling pathway
RBBP4	retinoblastoma binding protein 4	Present in protein complexes involved in: histone acetylation and chromatin assembly; chromatin remodelling and transcriptional repression associated with histone deacetylation; transcriptional silencing. Also binds directly to retinoblastoma protein to regulate cell proliferation.
HSPA8	heat shock 70kDa protein 8	Member of the heat shock protein 70 family, constitutively expressed. It functions as a chaperone, binding to nascent polypeptides to facilitate correct folding
SETDB1	SET domain, bifurcated 1	Histone methyltransferase which regulates histone methylation, gene silencing, and transcriptional repression
UBB	ubiquitin B	Required for ATP-dependent, nonlysosomal intracellular protein degradation of abnormal proteins and normal proteins with a rapid turnover, also involved in regulation of gene expression
ARNT	aryl hydrocarbon receptor nuclear translocator	Forms a complex with the ligand-bound aryl hydrocarbon receptor, and is required for induction of several enzymes that participate in xenobiotic metabolism
CXXC1	CXXC finger protein 1	Contains a CXXC motif within their DNA-binding domain, recognizes CpG sequences and regulate gene expression
MOGS	mannosyl-oligosaccharide glucosidase	Encodes the first enzyme in the N-linked oligosaccharide processing pathway
ACTG1	actin, gamma 1	Component of the cytoskeleton and mediator of internal cell motility found in non-muscle cells
KPNB1	karyopherin (importin) beta 1	Protein transporter of the importin beta family

To find functional similarities between these genes, Gene Ontology (GO) term enrichment analysis was performed using DAVID - Database for Annotation, Visualization and Integrated Discovery (D. W. Huang et al. 2008; D. W. Huang et al. 2009). Focusing on GO Terms relating to Biological processes, the most significant results relate to transcriptional regulation, cell cycle and cell proliferation.

Also significant is that, of this gene list, 3 genes were found in the GenAge database, which contains human genes related to ageing (de Magalhães et al. 2009):

- MDM2 – While there is no evidence of a direct link to ageing, this protein is known to inhibit TP53 (Momand et al. 1992), promoting cell growth. TP53 has been linked directly to ageing in mammalians (Cao et al. 2003; Bauer et al. 2005; Tyner et al. 2002). MDM2 has also been shown to be associated with cancer (G. L. Bond et al. 2004) and TP53-dependent apoptosis (Mendrysa et al. 2003).
- HSPA8 – This gene is involved in stress response. It has not been linked directly to ageing, nor do its levels vary with age; however age-related changes in stress response have been reported (Wu et al. 1993).
- UBB – Ubiquitin B regulates chromatin structure and stress response (R. T. Baker & Board 1987). Levels of heat shock-induced expression of two ubiquitin transcripts were shown to diminish with age in mice (Heydari et al. 1995). Ubiquitin B also seems to be associated with neurodegenerative diseases (van Leeuwen et al. 2002).

In the remaining top genes obtained in this analysis, there are a few involved in similar processes as these 3. ING1, for example, interacts with TP53, disrupting its regulation by MDM2 and inhibiting cell growth (Leung et al. 2002). CAPRIN1, on the other hand, is necessary for cell proliferation (B. Wang et al. 2005), although its specific function has not been determined. DNAJB6 is a molecular chaperone, as HSPA8, and has a role in inhibition of polyglutamine-dependent protein aggregation (Chuang et al. 2002). It has also been associated with cancer (Mitra et al. 2008). Other genes with functions in similar processes are RBBP4 and SETDB1, both associated with transcriptional regulation (Schultz et al. 2002; Gao et al. 2007; Lejon et al. 2011), with roles in cancer (H. Li et al. 2006; Ceol et al. 2011; Creekmore et al. 2008; Kong et al. 2007; Pacifico et al. 2007; Song et al. 2004) and neurodegenerative diseases (Ryu et al. 2006).

Considering these results, there is a few pathways that seem more likely to mediate CR and be the target of these drugs, such as cell cycle and cell growth/proliferation regulation, as well as stress response. It should be noted that all of these have already been associated with ageing

(Tyner et al. 2002; Cameron 1972; Kregel 2002; C.-K. Lee et al. 1999). Since there is also a significant association with transcriptional regulation, it would be useful to analyze which genes have their expression regulated by the genes on Table 9.

Furthermore, several of these genes have been associated with age-related diseases like cancer and neurodegenerative diseases and are part of common pathways, making them strong candidates for future experiments, as possible CR-related genes and mediators of the CR mimetics.

III.3.2 Analysis of significant genes for tested drugs vs. CR signature

While this analysis is similar to the previous one, the focus of this one was narrower, designed to elucidate the Cmap results for these specific drugs and how they might correlate to my experimental results. Furthermore, the experiments performed can only serve as evidence for these drugs' effect on lifespan and indication of their relation to CR. Analysing the genes involved may indicate possible targets and pathways mediating their effect on lifespan. However, as in the previous analysis, the results were different from what was expected (Table 10).

Table 10: Genes obtained from the comparison of the tested drugs with the CR signature

Drug	Gene	Drugs vs. CR[a]
Geldanamycin	HSPA8	CR↑ Drugs ↓
	DNAJB6 /// TMEM135[b]	CR↑ Drugs ↓
	SMPD2	CR ↑ Drugs ↑
	CRYZ	CR↑ Drugs ↓
	KPNB1	CR ↑ Drugs ↑
	POLR2A	CR ↑ Drugs ↑
	CXXC1	CR ↑ Drugs ↑
	UBB	CR↑ Drugs ↓
	PPP3CB	CR ↓ Drugs ↑
Rapamycin	HSPA8	CR ↑ Drugs ↑
	DNAJB6	CR ↑ Drugs ↑
	KPNB1	CR ↑ Drugs ↑
	CXXC1	CR ↑ Drugs ↑
	TFPI	CR ↓ Drugs ↓
LY-294002	HSPA8	CR ↑ Drugs ↑
	KPNB1	CR ↑ Drugs ↑
	MDM2	CR↑ Drugs ↓
	MOGS	CR ↑ Drugs ↑
	TFPI	CR ↓ Drugs ↓
Trichostatin A	SETDB1	CR ↑ Drugs ↑
	KPNB1	CR ↑ Drugs ↑
	ING1	CR↑ Drugs ↓
	FDFT1	CR ↓ Drugs ↓

[a]Compares behaviour of genes under CR with the effect of the drug. Arrow direction shows whether the gene is up or down-regulated in each situation.
[b]Different genes that bind to the same probe.

For Geldanamycin, most of the genes shown here have the opposite behaviour on CR as under the effect of this drug, while its positive enrichment in the Cmap would indicate the contrary result. On the other hand, for Rapamycin, LY-294002 and Trichostatin A, gene expression seems to vary in the same way under CR as under the effect of these drugs.

Most of the significant genes for each of these drugs were also significant genes in the global analysis and have already been discussed in the previous section. It should be noted that HSPA8, a gene that has been posited to be associated with age, is significant in the comparison of three of these drugs with the CR signature. The fact that this heat-shock protein is significant in this more specific analysis as well as in the global one, makes it a very strong candidate to be a mediator of these drugs effects on lifespan. The same can be said for others, as MDM2, ING1, SETDB1 and DNAJB6.

In the case of Geldanamycin and Trichostatin A, there are some genes whose expression has been significantly altered by this drug and that did not appear in the global analysis. None of them are known to be age-related, however their functions indicate that some of them can be relevant to the subject (Table 11).

Table 11: Function of some of the genes obtained in Table 10, not discussed before.

Gene Symbol	Gene Name	Function
SMPD2	sphingomyelin phosphodiesterase 2, neutral membrane (neutral sphingomyelinase)	Initially identified as a sphingomyelinase based on sequence similarity, subsequent studies showed that its biological function is less likely to be as a sphingomyelinase and instead as a lysophospholipase
CRYZ	crystallin, zeta (quinone reductase)	This gene encodes a taxon-specific crystallin protein which has NADPH-dependent quinone reductase activity distinct from other known quinone reductases
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	Largest subunit of RNA polymerase II, the polymerase responsible for synthesizing messenger RNA in eukaryotes
PPP3CB	protein phosphatase 3, catalytic subunit, beta isozyme	Part of the calcium signalling pathway; involved in T-cell activation
FDFT1	farnesyl-diphosphate farnesyltransferase 1	Membrane-associated enzyme located at a branch point in the mevalonate pathway; first specific enzyme in cholesterol biosynthesis, catalyzing the dimerization of two molecules of farnesyl diphosphate in a two-step reaction to form squalene

Most significant is the effects on PPP3CB, a gene involved in T-cell activation (Brogan et al. 2000). Ageing increases inflammation (Franceschi et al. 2000; Bruunsgaard et al. 2001) while CR has the opposite effect (C.-K. Lee et al. 2002; Higami et al. 2006), so the observed down-regulation of PP3CB by CR was to be expected (Table 10). Geldanamycin, on the other hand, up-regulates this gene (Table 10), which is once more consistent with the experimental data in Chapter II, but not with the Cmap results.

Of note is also FDFT1, a critical enzyme in the cholesterol biosynthesis (Do et al. 2009), that is down-regulated by CR and Trichostatin A (Table 10). Not only does it support the hypothesis that this drug is a CR mimetic (as observed experimentally), but it indicates that TSA may

reduce cholesterol which is one of the known CR benefits on human beings (Fontana et al. 2004).

In general, both these analysis have corroborated the experimental data of Chapter II. The Cmap results, on the other hand, are in direct contradiction with everything else, indicating that they may be mistaken. However, the candidates proposed by this tool seem to be significant, making it useful in this sort of analysis, even if the proposed effect is not consistent with experimental data. Another factor that might have made a difference is the CR signature used, containing only a small number of genes. However, several of the genes present in that signature are related to ageing and age-related diseases, which suggests that more accurate results could probably be obtained. The inconsistency seems then related to the specific Cmap algorithm used to calculate its results.

Chapter IV

Conclusion

IV.1 Final Conclusions and Perspectives

In conclusion, the Connectivity Map can be useful for selection of candidate drugs that induce a specific phenotype, in this case CR. While in this study the tested drugs had the opposite effect as predicted by the Cmap, the candidates obtained from this tool are still shown to yield interesting results in the context of their effect on lifespan and their relation to CR.

Specifically, 4 drugs out of the top results were chosen for lifespan testing. There was already some evidence in the literature that they were associated with lifespan, either by increasing or decreasing it. Three of them, Rapamycin, LY-294002 and Trichostatin A were known to increase lifespan (Babar et al. 1999; Harrison et al. 2009; Bjedov et al. 2010; Tao et al. 2004), however the Cmap predicted them induce the opposite phenotype of CR. Geldanamycin, on the other hand, is associated with induction of senescence in small cell lung cancer (Restall & Lorimer 2010), nonetheless the Cmap predicted it to be a CR mimetic. This prediction might be related to the anti-cancer effect of CR, since Geldanamycin is known to have anti-cancer properties (Fukuyo et al. 2010). This function might perhaps be the reason it induces a similar gene expression profile to CR.

These lifespan assays were carried out in *C. elegans*, both N2 (fed AD) and *eat-2* (naturally under CR). The experimental data was in accordance to the literature, with Rapamycin, LY-294002 and Trichostatin A increasing lifespan of N2 animals, by 19.2%, 19.2% and 11.5% of median survival, respectively. Yet they did not have the same effect on the *eat-2* mutants, suggesting that this lifespan increase occurs through the same process as CR. While Rapamycin and LY-294002 were suspected to be CRM before, to my knowledge Trichostatin A effect on lifespan had not been linked to CR before.

Conversely, Geldanamycin had no significant effect on the N2 worms but mange to decrease *eat-2* lifespan, with its median survival reduced by 26.9%. Since *eat-2*'s increased lifespan is attributed to CR, it is likely that Geldanamycin affects the same pathways as CR, cancelling the lifespan extension it induces.

The results obtained with the 4 tested drugs are consistent with previous studies on their function. However they are in direct opposition with the Cmap predictions.

For better understanding of the Cmap output, its database was analyzed to determine the most significant genes in the comparison between the query CR signature and the top drugs and how their expression was altered in both cases. A similar analysis was done for the 4

tested drugs. The results support the experimental data, with most of the significant genes for Rapamycin, LY-294002 and TSA having the same behaviour under CR and under the effect o these drugs, while for Geldanamycin the opposite happened. In the analysis involving all top drugs, the positive enrichment drugs yielded similar results to Geldanamycin while the negative enrichment ones show results akin to Rapamycin, LY-294002 and TSA.

These results seem to indicate that the Cmap might not show the true effect of these drugs, but the opposite one.

It should be noted that DMSO can extend lifespan in *C. elegans*, in a *daf-16* dependent manner (Xiangming Wang et al. 2010), although I did not know this at the time these experiments were performed. Since the drugs tested were dissolved in this solvent, this might be affecting the results obtained. These experiments would need to be repeated using DMSO in the controls or an alternate solvent.

Another factor that might influence the results is the method by which these worms were subjected to CR. There is evidence that indicates that different methods of CR can trigger at least partly different pathways, depending on how severe the degree of CR (Kenyon 2010; Bishop & Guarente 2007a). Therefore, it would be important to assess whether different CR-inducing methods would yield different results. This might also allow for better understanding of the pathways required for different CR levels.

Despite these caveats, these results demonstrate that Rapamycin, LY-294002 and Trichostatin A are capable of inducing a CR-like state and therefore should be considered as CRM candidates for testing in other model species, with the ultimate goal of determining if they can be used as CRMs for human beings. These future studies should also take into account possible undesirable side effects of these drugs. For example, Rapamycin has immunosuppressant effects and metformin (another CRM candidate, but not part of this study) can cause gastrointestinal upset (Cabreiro & Gems 2010). It is also important to study the mechanisms involved in the action of these drugs. For Rapamycin and LY-294002, the drug targets implicated are already suspected to be TOR and PI-3K, respectively. For Trichostatin A, however, such a target and the corresponding signalling cascade are unknown, as this drug, had never been connected to CR before.

Most surprisingly, they reveal that Geldanamycin is capable of completely abrogating the lifespan increase induced by CR. Further studies are necessary to determine the pathways

involved in this effect, but their understanding could lead to a great improvement in our understanding of the mechanisms of CR.

Bibliography

Altun, Z.F. & Hall, D.H., 2009. Introduction. In *WormAtlas*.

Amador-Noguez, D. et al., 2007. Alterations in xenobiotic metabolism in the long-lived Little mice. *Aging Cell*, 6(4), pp.453–470.

An, J.H. & Blackwell, T.K., 2003. SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes & development*, 17(15), pp.1882–93.

Anderson, R.M., Shanmuganayagam, D. & Weindruch, R., 2009. Caloric restriction and aging: studies in mice and monkeys. *Toxicologic Pathology*, 37(1), pp.47–51.

Anisimov, V.N. et al., 2008. Metformin slows down aging and extends life span of female SHR mice. *Cell Cycle (Georgetown, Tex.)*, 7(17), pp.2769–73.

Arantes-Oliveira, N., Berman, J.R. & Kenyon, C., 2003. Healthy Animals with Extreme Longevity. *Science*, 302(5645), p.611.

Avery, L., 2003. Food transport in the C. elegans pharynx. *Journal of Experimental Biology*, 206(14), pp.2441–2457.

Avery, L., 1993. The genetics of feeding in Caenorhabditis elegans. *Genetics*, 133, pp.897–917.

Ayyadevara, S. et al., 2008. Remarkable longevity and stress resistance of nematode PI3K-null mutants. *Aging Cell*, 7(1), pp.13–22.

Babar, P. et al., 1999. P13-kinase inhibition induces dauer formation, thermotolerance and longevity in C. elegans. *Neurobiology of Aging*, 20(5), pp.513–9.

Baker, R.T. & Board, P.G., 1987. The human ubiquitin gene family: structure of a gene and pseudogenes from the Ub B subfamily. *Nucleic Acids Research*, 15(2), pp.443–463.

Bartke, A., 2005. Minireview: Role of the Growth Hormone/Insulin-Like Growth Factor System in Mammalian Aging. *Endocrinology*, 146(9), pp.3718–3723.

Bates, E.A. et al., 2006. Differential contributions of Caenorhabditis elegans histone deacetylases to huntingtin polyglutamine toxicity. *The Journal of Neuroscience*, 26(10), pp.2830–8.

Bauer, J.H. et al., 2005. Neuronal Expression of p53 Dominant-Negative Proteins in Adult Drosophila melanogaster Extends Life Span. *Current biology*, 15(22), pp.2063–2068.

Beckman, K.B. & Ames, B.N., 1998. The Free Radical Theory of Aging Matures. *Physiological Reviews*, 78(2), pp.547–581.

Biggs, W.H. et al., 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proceedings of the National Academy of Sciences of the United States of America*, 96(13), pp.7421–7426.

Bishop, N.A. & Guarente, L., 2007a. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. *Nature Reviews Genetics*, 8(11), pp.835–44.

Bishop, N.A. & Guarente, L., 2007b. Two neurons mediate diet-restriction-induced longevity in C. elegans. *Nature*, 447(7144), pp.545–9.

Bjedov, I. et al., 2010. Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metabolism*, 11(1), pp.35–46.

Blagosklonny, M.V., 2006. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. *Cell Cycle*, 5(18), pp.2087–2102.

Blagosklonny, M.V., 2009. Aging-suppressants: Cellular senescence (hyperactivation) and its pharmacologic deceleration. *Cell Cycle*, 8(12), pp.1883–1887.

Blagosklonny, M.V., 2010. Calorie restriction: Decelerating mTOR-driven aging from cells to organisms (including humans). *Cell Cycle*, 9(4), pp.683–688.

Bond, G.L. et al., 2004. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell*, 119(5), pp.591–602.

Brenner, S., 1974. The genetics of Caenorhabditis elegans. *Genetics*, pp.71–94.

Brogan, I.J., Pravica, V. & Hutchinson, I.V., 2000. Genetic conservation of the immunophilin-binding domains of human calcineurin A1 and A2. *Transplant Immunology*, 8(2), pp.139–141.

Broughton, S.J. et al., 2005. Longer lifespan, altered metabolism, and stress resistance in Drosophila from ablation of cells making insulin-like ligands. *Proceedings of the National Academy of Sciences of the United States of America*, 102(8), pp.3105–3110.

Brown-Borg, H.M., 2007. Hormonal regulation of longevity in mammals. *Ageing Research Reviews*, 6(1), pp.28–45.

Brunet, A. et al., 1999. Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor. *Cell*, 96(6), pp.857–868.

Bruunsgaard, H., Pedersen, M. & Pedersen, B.K., 2001. Aging and proinflammatory cytokines. *Current Opinion in Hematology*, 8(3).

Burns, A.R. et al., 2010. A predictive model for drug bioaccumulation and bioactivity in Caenorhabditis elegans. *Nature Chemical Biology*, 6(7), pp.549–57.

de Cabo, R. et al., 2003. An in vitro model of caloric restriction. *Experimental Gerontology*, 38(6), pp.631–639.

Cabreiro, F. & Gems, D., 2010. Treating aging: progress toward dietary restriction mimetics. *F1000 Biology Reports*, 2(October), p.76.

Cameron, I.L., 1972. Cell Proliferation and Renewal in Aging Mice. *Journal of Gerontology*, 27(2), pp.162–172.

Cao, L. et al., 2003. Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. *Genes & development*, 17(2), pp.201–13.

Ceol, C.J. et al., 2011. The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset. *Nature*, 471(7339), pp.513–517.

Chippindale, A.K. et al., 1993. Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. *Journal of Evolutionary Biology*, 6(2), pp.171–193.

Cho, S.C. et al., 2010. DDS, 4,4'-diaminodiphenylsulfone, extends organismic lifespan. *Proceedings of the National Academy of Sciences of the United States of America*, 107(45), pp.19326–31.

Choy, S.W. et al., 2007. C. elegans SIN-3 and its associated HDAC corepressor complex act as mediators of male sensory ray development. *Biochemical and Biophysical Research Communications*, 358(3), pp.802–7.

Chuang, J.-Z. et al., 2002. Characterization of a Brain-enriched Chaperone, MRJ, That Inhibits Huntingtin Aggregation and Toxicity Independently. *Journal of Biological Chemistry*, 277(22), pp.19831–19838.

Clancy, D.J. et al., 2002. Dietary Restriction in Long-Lived Dwarf Flies. *Science*, 296(5566), p.319.

Cohen, E. et al., 2006. Opposing Activities Protect Against Age-Onset Proteotoxicity. *Science*, 313(5793), pp.1604–1610.

Cohen, E. et al., 2009. Reduced IGF-1 signaling delays age-associated proteotoxicity in mice. *Cell*, 139(6), pp.1157–69.

Colman, R.J. et al., 2009. Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys. *Science*, 325(5937), pp.201–204.

Cooper, T.M. et al., 2004. Effect of caloric restriction on life span of the housefly, Musca domestica. *The FASEB Journal*, 18(13), pp.1591–1593.

Cox, G.N., Kusch, M. & Edgar, R.S., 1981. Cuticle of Caenorhabditis elegans: its isolation and partial characterization. *The Journal of Cell biology*, 90(1), pp.7–17.

Creekmore, A.L. et al., 2008. The role of retinoblastoma-associated proteins 46 and 48 in estrogen receptor α mediated gene expression. *Molecular and Cellular Endocrinology*, 291(1–2), pp.79–86.

Dhahbi, J.M. et al., 2005. Identification of potential caloric restriction mimetics by microarray profiling. *Physiological genomics*, 23(3), pp.343–50.

Dillin, A., Crawford, D.K. & Kenyon, C., 2002. Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science (New York, N.Y.)*, 298(5594), pp.830–4.

Do, R. et al., 2009. Squalene synthase: a critical enzyme in the cholesterol biosynthesis pathway. *Clinical Genetics*, 75(1), pp.19–29.

Fire, A. et al., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391, pp.806–811.

Fontana, L. et al., 2004. Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 101(17), pp.6659–6663.

Fontana, L. & Klein, S., 2007. Aging, Adiposity, and Calorie Restriction. *JAMA: The Journal of the American Medical Association*, 297(9), pp.986–994.

Fontana, L., Partridge, L. & Longo, V.D., 2010. Extending healthy life span--from yeast to humans. *Science*, 328(5976), pp.321–6.

Franceschi, C. et al., 2000. Inflamm-aging: An Evolutionary Perspective on Immunosenescence. *Annals of the New York Academy of Sciences*, 908(1), pp.244–254.

Fujikake, N. et al., 2008. Heat shock transcription factor 1-activating compounds suppress polyglutamine-induced neurodegeneration through induction of multiple molecular chaperones. *Journal of Biological Chemistry*, 283(38), p.26188.

Fukuyo, Y., Hunt, C.R. & Horikoshi, N., 2010. Geldanamycin and its anti-cancer activities. *Cancer Letters*, 290(1), pp.24–35.

Furuyama, T. et al., 2000. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochemical Journal*, 349, pp.629–634.

Félix, M.-A., 2008. RNA interference in nematodes and the chance that favored Sydney Brenner. *Journal of biology*, 7(9), p.34.

Gao, H. et al., 2007. Akt/PKB interacts with the histone H3 methyltransferase SETDB1 and coordinates to silence gene expression. *Molecular and Cellular Biochemistry*, 305(1), pp.35–44.

Garcia, A.M. et al., 2008. Effect of Ames dwarfism and caloric restriction on spontaneous DNA mutation frequency in different mouse tissues. *Mechanisms of Ageing*, 129(9), pp.528–533.

Gaudet, J. & Mango, S.E., 2002. Regulation of organogenesis by the Caenorhabditis elegans FoxA protein PHA-4. *Science (New York, N.Y.)*, 295(5556), pp.821–5.

Gems, D., 2011. Tragedy and delight: the ethics of decelerated ageing. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 366(1561), pp.108–112.

Gems, D. & Doonan, R., 2009. Antioxidant defense and aging in C. elegans: Is the oxidative damage theory of aging wrong? *Cell Cycle*, 8(11), pp.1681–1687.

Giannakou, M.E. et al., 2007. Dynamics of the action of dFOXO on adult mortality in Drosophila. *Aging cell*, 6(4), pp.429–38.

Giannakou, M.E., Goss, M. & Partridge, L., 2008. Role of dFOXO in lifespan extension by dietary restriction in Drosophila melanogaster: not required, but its activity modulates the response. *Aging Cell*, 7(2), pp.187–98.

Greer, E.L. et al., 2007. An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. *Current biology*, 17(19), pp.1646–56.

Greer, E.L. & Brunet, A., 2009. Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in C. elegans. *Aging Cell*, 8(2), pp.113–127.

Grönke, S. et al., 2010. Molecular Evolution and Functional Characterization of Drosophila Insulin-Like Peptides. *PLoS Genetics*, 6(2), p.e1000857.

Guarente, L. & Kenyon, C., 2000. Genetic pathways that regulate ageing in model organisms. *Nature*, 408(6809), pp.255–262.

Gustavo, B., 2004. Free radicals and aging. *Trends in Neurosciences*, 27(10), pp.595–600.

Hansen, M. et al., 2008. A Role for Autophagy in the Extension of Lifespan by Dietary Restriction in C. elegans. *PLoS Genetics*, 4(2), p.e24.

Harman, D., 1956. Aging: A Theory Based on Free Radical and Radiation Chemistry. *Journal of Gerontology*, 11(3), pp.298–300.

Harrison, D.E. et al., 2009. Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature*, 460(7253), pp.392–5.

Heydari, A.R., Conrad, C.C. & Richardson, A., 1995. Expression of Heat Shock Genes in Hepatocytes is Affected by Age and Food Restriction in Rats. *The Journal of Nutrition*, 125(3), pp.410–418.

Higami, Y. et al., 2006. Energy Restriction Lowers the Expression of Genes Linked to Inflammation, the Cytoskeleton, the Extracellular Matrix, and Angiogenesis in Mouse Adipose Tissue. *The Journal of Nutrition*, 136(2), pp.343–352.

Houthoofd, K. et al., 2003. Life extension via dietary restriction is independent of the Ins/IGF-1 signalling pathway in Caenorhabditis elegans. *Experimental Gerontology*, 38(9), pp.947–954.

Huang, D.W., Sherman, B.T. & Lempicki, R.A., 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Research*, 37(1), pp.1–13.

Huang, D.W., Sherman, B.T. & Lempicki, R.A., 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, 4(1), pp.44–57.

Ikeno, Y. et al., 2009. Reduced Incidence and Delayed Occurrence of Fatal Neoplastic Diseases in Growth Hormone Receptor/Binding Protein Knockout Mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 64A(5), pp.522–529.

Ingram, D.K. et al., 2006. Calorie restriction mimetics: an emerging research field. *Aging cell*, 5(2), pp.97–108.

Inoki, K. & Guan, K.-L., 2006. Complexity of the TOR signaling network. *Trends in Cell Biology*, 16(4), pp.206–212.

Inoue, H. et al., 2005. The C. elegans p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. *Genes & development*, 19(19), pp.2278–83.

Itoh, K. et al., 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and Biophysical Research Communications*, 236(2), pp.313–22.

Jia, K., Chen, D. & Riddle, D.L., 2004. The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. *Development*, 131(16), pp.3897–3906.

Jiang, J.C. et al., 2000. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *The FASEB Journal*.

Johnson, F.B., Sinclair, D.A. & Guarente, L., 1999. Molecular Biology of Aging. *Cell*, 96(2), pp.291–302.

Kaeberlein, M. et al., 2005. Regulation of Yeast Replicative Life Span by TOR and Sch9 in Response to Nutrients. *Science*, 310(5751), pp.1193–1196.

Kaeberlein, T.L. et al., 2006. Lifespan extension in Caenorhabditis elegans by complete removal of food. *Aging Cell*, 5(6), pp.487–494.

Kalender, A. et al., 2010. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell metabolism*, 11(5), pp.390–401.

Kapahi, P. et al., 2004. Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway. *Current Biology*, 14(10), pp.885–890.

Kapahi, P. & Zid, B.M., 2004. TOR Pathway: Linking Nutrient Sensing to Life Span. *Science of Aging Knowledge Environment*, 2004(36), p.pe34.

Kenyon, C. et al., 1993. A C. elegans mutant that lives twice as long as wild type. *Nature*, 366(6454), pp.461–464.

Kenyon, C., 2010. The genetics of ageing. *Nature*, 464(7288), pp.504–512.

Kimble, J. & Hirsh, D., 1979. The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. *Developmental Biology*, 70(2), pp.396–417.

Kirkwood, T.B.L. & Shanley, D.P., 2005. Food restriction, evolution and ageing. *Mechanisms of Ageing and Development*, 126(9), pp.1011–1016.

Klass, M.R., 1977. Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. *Mechanisms of Ageing and Development*, 6, pp.413–429.

Kong, L. et al., 2007. RbAp48 is a critical mediator controlling the transforming activity of human papillomavirus type 16 in cervical cancer. *The Journal of Biological Chemistry*, 282(36), pp.26381–91.

Kops, G.J.P.L. et al., 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature*, 398(6728), pp.630–634.

Kregel, K.C., 2002. Invited Review: Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology*, 92(5), pp.2177–2186.

Lakowski, B. & Hekimi, S., 1998. The genetics of caloric restriction in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America*, 95(22), pp.13091–13096.

Lamb, J. et al., 2006. The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. *Science (New York, N.Y.)*, 313(5795), pp.1929–35.

Lapointe, J. & Hekimi, S., 2010. When a theory of aging ages badly. *Cellular and Molecular Life Sciences*, 67(1), pp.1–8.

Lashinger, L.M. et al., 2011. Rapamycin Partially Mimics the Anticancer Effects of Calorie Restriction in a Murine Model of Pancreatic Cancer . *Cancer Prevention Research* , 4 (7), pp.1041–1051.

Lee, C., Raffaghello, L. & Longo, V.D., 2012. Starvation, detoxification, and multidrug resistance in cancer therapy. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy*, 15(1-2), pp.114–122.

Lee, C.-K. et al., 1999. Gene Expression Profile of Aging and Its Retardation by Caloric Restriction. *Science*, 285(5432), pp.1390–1393.

Lee, C.-K. et al., 2002. Transcriptional profiles associated with aging and middle age-onset caloric restriction in mouse hearts. *Proceedings of the National Academy of Sciences of the United States of America*, 99(23), pp.14988–93.

van Leeuwen, F.W. et al., 2002. +1 Proteins and aging. *The International Journal of Biochemistry & Cell Biology*, 34(11), pp.1502–1505.

Lejon, S. et al., 2011. Insights into Association of the NuRD Complex with FOG-1 from the Crystal Structure of an RbAp48·FOG-1 Complex. *Journal of Biological Chemistry*, 286(2), pp.1196–1203.

Leung, K.M. et al., 2002. The Candidate Tumor Suppressor ING1b Can Stabilize p53 by Disrupting the Regulation of p53 by MDM2. *Cancer Research*, 62(17), pp.4890–4893.

Li, H. et al., 2006. The Histone Methyltransferase SETDB1 and the DNA Methyltransferase DNMT3A Interact Directly and Localize to Promoters Silenced in Cancer Cells. *Journal of Biological Chemistry*, 281(28), pp.19489–19500.

Lindblom, T.H. & Dodd, A.K., 2006. Xenobiotic Detoxification in the Nematode Caenorhabditis elegans. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, 305A(9), pp.720–730.

de Magalhães, J.P. et al., 2009. The Human Ageing Genomic Resources: online databases and tools for biogerontologists. *Aging Cell*, 8(1), pp.65–72.

de Magalhães, J.P. & Costa, J., 2009. A database of vertebrate longevity records and their relation to other life-history traits. *Journal of Evolutionary Biology*, 22(8), pp.1770–1774.

Mair, W. et al., 2003. Demography of dietary restriction and death in Drosophila. *Science (New York, N.Y.)*, 301(5640), pp.1731–3.

Mair, W. & Dillin, A., 2008. Aging and survival: the genetics of life span extension by dietary restriction. *Annual Review of Biochemistry*, 77, pp.727–54.

Mango, S.E., Lambie, E.J. & Kimble, J., 1994. The pha-4 gene is required to generate the pharyngeal primordium of Caenorhabditis elegans. *Development (Cambridge, England)*, 120(10), pp.3019–31.

Matuoka, K., Chen, K.Y. & Takenawa, T., 2003. A positive role of phosphatidylinositol 3-kinase in aging phenotype expression in cultured human diploid fibroblasts. *Archives of Gerontology and Geriatrics*, 36(3), pp.203–19.

McCay, C.M., Crowell, M.F. & Maynard, L.A., 1935. The effect of retarded growth upon the length of life span and upon the ultimate body size. *Journal of Nutrition*, 10, pp.63–79.

Mckay, J.P. et al., 2004. eat-2 and eat-18 are required for nicotinic neurotransmission in the Caenorhabditis elegans pharynx. *Genetics*, 166, pp.161–169.

Mendrysa, S.M. et al., 2003. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. *Molecular and Cellular Biology*, 23(2), pp.462–463.

Min, K.-J. et al., 2008. Drosophila lifespan control by dietary restriction independent of insulin-like signaling. *Aging Cell*, 7(2), pp.199–206.

Mitra, A. et al., 2008. Large isoform of MRJ (DNAJB6) reduces malignant activity of breast cancer. *Breast Cancer Research : BCR*, 10(2), p.R22.

Momand, J. et al., 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*, 69(7), pp.1237–1245.

Moskalev, A.A. & Shaposhnikov, M.V., 2010. Pharmacological inhibition of phosphoinositide 3 and TOR kinases improves survival of Drosophila melanogaster. *Rejuvenation Research*, 13(2-3), pp.246–7.

Nogueira, L.M. et al., 2012. Calorie Restriction and Rapamycin Inhibit MMTV-Wnt-1 Mammary Tumor Growth in a Mouse Model of Postmenopausal Obesity. *Endocrine-Related Cancer*, 19, pp.57–68.

Onken, B. & Driscoll, M., 2010. Metformin induces a dietary restriction-like state and the oxidative stress response to extend C. elegans healthspan via AMPK, LKB1, and SKN-1. *PloS one*, 5(1), p.e8758.

Pacifico, F. et al., 2007. RbAp48 is a target of nuclear factor-kappaB activity in thyroid cancer. *The Journal of Clinical Endocrinology and Metabolism*, 92(4), pp.1458–66.

Panowski, S.H. et al., 2007. PHA-4/Foxa mediates diet-restriction-induced longevity of C. elegans. *Nature*, 447(7144), pp.550–555.

Partridge, L. & Gems, D., 2002. Mechanisms of aging: public or private? *Nature Reviews Genetics*, 3(3), pp.165–175.

Patel, N.V. et al., 2005. Caloric restriction attenuates Abeta-deposition in Alzheimer transgenic models. *Neurobiology of Aging*, 26(7), pp.995–1000.

Piper, M.D. et al., 2008. Separating cause from effect: how does insulin/IGF signalling control lifespan in worms, flies and mice? *Journal of Internal Medicine*, 263(2), pp.179–191.

Powers III, R.W. et al., 2006. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes & Development*, 20(2), pp.174–184.

Raffaghello, L. et al., 2010. Fasting and differential chemotherapy protection in patients. *Cell Cycle*, 9(22), pp.4474–4476.

Raffaghello, L. et al., 2008. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proceedings of the National Academy of Sciences of the United States of America*, 105(24), pp.8215–20.

Restall, I.J. & Lorimer, I.A.J., 2010. Induction of premature senescence by hsp90 inhibition in small cell lung cancer. *PloS ONE*, 5(6), p.e11076.

Ryu, H. et al., 2006. ESET/SETDB1 gene expression and histone H3 (K9) trimethylation in Huntington's disease. *Proceedings of the National Academy of Sciences of the United States of America*, 103(50), pp.19176–19181.

Sahra, I.B. et al., 2011. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer research*, 71(13), pp.4366–72.

Schultz, D.C. et al., 2002. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes & development*, 16(8), pp.919–32.

Selman, C. et al., 2008. Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *The FASEB Journal*, 22(3), pp.807–818.

Selman, C. et al., 2009. Ribosomal Protein S6 Kinase 1 Signaling Regulates Mammalian Life Span. *Science*, 326(5949), pp.140–144.

Skantar, A.M. et al., 2005. Effects of geldanamycin on hatching and juvenile motility in Caenorhabditis elegans and Heterodera glycines. *Journal of Chemical Ecology*, 31(10), pp.2481–91.

Sohal, R.S. & Weindruch, R., 1996. Oxidative Stress, Caloric Restriction, and Aging. *Science*, 273(5271), pp.59–63.

Song, H. et al., 2004. Genes encoding Pir51, Beclin 1, RbAp48 and aldolase b are up or down-regulated in human primary hepatocellular carcinoma. *World Journal of Gastroenterology : WJG*, 10(4), pp.509–13.

Steffen, K.K. et al., 2008. Yeast Life Span Extension by Depletion of 60S Ribosomal Subunits Is Mediated by Gcn4. *Cell*, 133(2), pp.292–302.

Steinkraus, K.A. et al., 2008. Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in Caenorhabditis elegans. *Aging Cell*, 7(3), pp.394–404.

Stiernagle, T., 2006. Maintenance of C. elegans. In *WormBook : the online review of C. elegans biology*. pp. 1–11.

Sulston, J.E. & Horvitz, H.R., 1977. Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. *Developmental Biology*, 56(1), pp.110–156.

Tao, D. et al., 2004. Trichostatin A extends the lifespan of Drosophila melanogaster by elevating hsp22 expression. *Acta Bochimica et Biophysica Sinica*, 36(9), pp.618–22.

Tatar, M. et al., 2001. A Mutant Drosophila Insulin Receptor Homolog That Extends Life-Span and Impairs Neuroendocrine Function. *Science*, 292(5514), pp.107–110.

Tullet, J.M.A. et al., 2008. Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans. *Cell*, 132(6), pp.1025–38.

Tyner, S.D. et al., 2002. p53 mutant mice that display early ageing-associated phenotypes. *Nature*, 415(6867), pp.45–53.

United Nations, 2001. WORLD POPULATION AGEING : 1950-2050. *Population (English Edition)*.

Vellai, T. et al., 2003. Genetics: Influence of TOR kinase on lifespan in C. elegans. *Nature*, 426(6967), p.620.

Walker, A.K. et al., 2000. A conserved transcription motif suggesting functional parallels between Caenorhabditis elegans SKN-1 and Cap'n'Collar-related basic leucine zipper proteins. *The Journal of Biological Chemistry*, 275(29), pp.22166–71.

Walker, G.A. et al., 2005. Dietary restriction in C. elegans: from rate-of-living effects to nutrient sensing pathways. *Mechanisms of Ageing and Development*, 126(9), pp.929–37.

Wang, B., David, M.D. & Schrader, J.W., 2005. Absence of Caprin-1 Results in Defects in Cellular Proliferation. *The Journal of Immunology*, 175(7), pp.4274–4282.

Wang, Xiangming et al., 2010. Lifespan extension in Caenorhabditis elegans by DMSO is dependent on sir-2.1 and daf-16. *Biochemical and biophysical research communications*, 400(4), pp.613–8.

Wei, M. et al., 2009. Tor1/Sch9-Regulated Carbon Source Substitution Is as Effective as Calorie Restriction in Life Span Extension. *PLoS Genetics*, 5(5), p.e1000467.

Weindruch, R. & Walford, R.L., 1982. Dietary restriction in mice beginning at one year of age: effect on life-span and spontaneous cancer incidence. *Science of Aging Knowledge Environment*, 215, pp.1415–1418.

Weinkove, D. et al., 2006. Long-term starvation and ageing induce AGE-1/PI 3-kinase-dependent translocation of DAF-16/FOXO to the cytoplasm. *BMC Biology*, 4, p.1.

Wu, B. et al., 1993. The Effect of Age on the Synthesis of Two Heat Shock Proteins in the HSP70 Family. *Journal of Gerontology*, 48(2), pp.B50–B56.

Wullschleger, S., Loewith, R. & Hall, M.N., 2006. TOR Signaling in Growth and Metabolism. *Cell*, 124(3), pp.471–484.

Yamamoto, R. & Tatar, M., 2011. Insulin receptor substrate chico acts with the transcription factor FOXO to extend Drosophila lifespan. *Aging Cell*, 10(4), pp.729–732.

Appendixes

Appendix 1

Table 12: All genes from the comparison of positive enrichment drugs with the CR signature

Gene	N[a]	CR vs. Drugs[b]
MDM2	12	CR↑ Drugs ↓
CAPRIN1	10	CR ↓ Drugs ↑
DNAJB6	9	CR ↑ Drugs ↓
TFPI	9	CR ↓ Drugs ↑
DNAJB6 /// TMEM135[c]	8	CR ↑ Drugs ↓
ING1	8	CR ↑ Drugs ↓
RBBP4	8	CR ↓ Drugs ↑
HSPA8	7	CR ↑ Drugs ↓
SETDB1	7	CR ↑ Drugs ↑
UBB	7	CR ↑ Drugs ↓
KPNB1	6	CR ↑ Drugs ↑
CXXC1	6	CR ↑ Drugs ↑
KPNB1	5	CR↑ Drugs ↓
POLR2A	5	CR ↑ Drugs ↑
POLR2A	5	CR↑ Drugs ↓
MOGS	5	CR ↑ Drugs ↑
MAP7	5	CR ↓ Drugs ↑
MAP7	5	CR ↓ Drugs ↓
PPP3CB	5	CR ↓ Drugs ↑
SMPD2	4	CR ↑ Drugs ↑
SETDB1	4	CR↑ Drugs ↓
CRYZ	4	CR↑ Drugs ↓
ING1	4	CR ↑ Drugs ↑
ARNT	4	CR↑ Drugs ↓
CA4	4	CR↑ Drugs ↓
ACTG1	4	CR ↓ Drugs ↑
MDM2	3	CR ↑ Drugs ↑
ARNT	3	CR ↑ Drugs ↑
MOGS	3	CR↑ Drugs ↓
RPL23	3	CR ↓ Drugs ↑
RPS8 /// RPS8P8	2	CR↑ Drugs ↓
JAK1	2	CR↑ Drugs ↓
SUCLA2	2	CR ↑ Drugs ↑
CA4	2	CR ↑ Drugs ↑
TFPI	2	CR ↓ Drugs ↓
RBBP4	2	CR ↓ Drugs ↓
FDFT1	2	CR ↓ Drugs ↑
FDFT1	2	CR ↓ Drugs ↓
RPS8 /// RPS8P8	1	CR ↑ Drugs ↑
HSPA8	1	CR ↑ Drugs ↑
DNAJB6 /// TMEM135[c]	1	CR ↑ Drugs ↑
SMPD2	1	CR↑ Drugs ↓
CRYZ	1	CR ↑ Drugs ↑
SUCLA2	1	CR↑ Drugs ↓
CXXC1	1	CR↑ Drugs ↓
UBB	1	CR ↑ Drugs ↑
LAMA1	1	CR ↓ Drugs ↑
RPL23	1	CR ↓ Drugs ↓
PPP3CB	1	CR ↓ Drugs ↓

[a]N stands for the number of drugs with positive enrichment (out of a total of 14) in which the gene was either in the top or bottom 10.

[b]Compares behavior of genes under CR with the effect of the drugs with positive enrichment. Arrow direction shows whether the gene is up or down-regulated in each situation.

[c]Different genes that bind to the same probe.

Genes that appear twice on the table show both behaviors: some drugs cause it to be up-regulated, while others down-regulate it.

Table 13: All genes from the comparison of negative enrichment drugs with the CR signature

Gene	N[a]	CR vs. Drugs[b]
ARNT	9	CR ↑ Drugs ↑
TFPI	9	CR ↓ Drugs ↓
MDM2	8	CR↑ Drugs ↓
CXXC1	8	CR ↑ Drugs ↑
MOGS	8	CR ↑ Drugs ↑
ACTG1	8	CR ↓ Drugs ↓
SETDB1	7	CR ↑ Drugs ↑
ING1	7	CR ↑ Drugs ↑
DNAJB6 /// TMEM135	6	CR ↑ Drugs ↑
KPNB1	6	CR ↑ Drugs ↑
CA4	6	CR↑ Drugs ↓
SMPD2	5	CR↑ Drugs ↓
ARNT	5	CR↑ Drugs ↓
RPL23	5	CR ↓ Drugs ↓
RBBP4	5	CR ↓ Drugs ↓
MAP7	5	CR ↓ Drugs ↑
HSPA8	4	CR ↑ Drugs ↑
POLR2A	4	CR ↑ Drugs ↑
MDM2	4	CR ↑ Drugs ↑
UBB	4	CR ↑ Drugs ↑
MAP7	4	CR ↓ Drugs ↓
CAPRIN1	4	CR ↓ Drugs ↑
SMPD2	3	CR ↑ Drugs ↑
POLR2A	3	CR↑ Drugs ↓
RPS8 /// RPS8P8	2	CR↑ Drugs ↓
DNAJB6 /// TMEM135	2	CR↑ Drugs ↓
DNAJB6	2	CR ↑ Drugs ↑
DNAJB6	2	CR↑ Drugs ↓
SETDB1	2	CR↑ Drugs ↓
CRYZ	2	CR ↑ Drugs ↑
CRYZ	2	CR↑ Drugs ↓
KPNB1	2	CR↑ Drugs ↓
WRNIP1	2	CR↑ Drugs ↓
ING1	2	CR↑ Drugs ↓
SUCLA2	2	CR ↑ Drugs ↑
CXXC1	2	CR↑ Drugs ↓
UBB	2	CR↑ Drugs ↓
PPP3CB	2	CR ↓ Drugs ↑
FDFT1	2	CR ↓ Drugs ↑
FDFT1	2	CR ↓ Drugs ↓
HSPA8	1	CR↑ Drugs ↓
RPS8	1	CR↑ Drugs ↓
JAK1	1	CR↑ Drugs ↓
HSD17B4	1	CR ↑ Drugs ↑
TFPI	1	CR ↓ Drugs ↑
RPL23	1	CR ↓ Drugs ↑
RBBP4	1	CR ↓ Drugs ↑
CAPRIN1	1	CR ↓ Drugs ↓
PPP3CB	1	CR ↓ Drugs ↓

[a]N stands for the number of drugs with negative enrichment (out of a total of 12) in which the gene was either in the top or bottom 10.

[b]Compares behavior of genes under CR with the effect of the drugs with negative enrichment. Arrow direction shows whether the gene is up or down-regulated in each situation.

[c]Different genes that bind to the same probe.

Genes that appear twice on the table show both behaviors: some drugs cause it to be up-regulated, while others down-regulate it.